The Effects of Zinc Ions on Bone Growth, Modeling and Remodeling

Ph.D. Thesis

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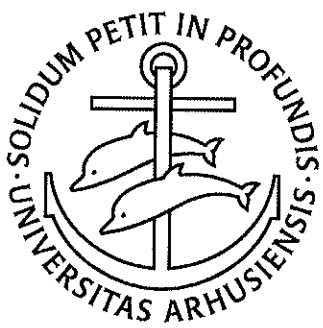

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University of Aarhus
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Department of Orthopaedic Surgery
University Hospital of Aarhus

2005

Denne afhandling er af det Sundhedsvidenskabelige Fakultet ved Aarhus Universitet den 31. maj 2005 antaget til offentligt forsvar for den medicinske Ph.D. grad.

Forsvaret finder sted fredag den 12. august 2005, kl. 14.00 i det Blå Auditorium, Victor Albech bygningen, Aarhus Universitet.

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The thesis is based on the following papers:

I. The Positive Effects of Zinc on Skeletal Strength in Growing Rats. Ovesen J, Møller-Madsen B, Thomsen JS, Danscher G and Mosekilde Li. Bone 2001; 29(6):565–570.
II. Autometallographic Tracing of Zinc Ions in Growing Bone. Ovesen J, Danscher G, Thomsen JS, Mosekilde Li and Møller-Madsen B. Journal of Musculoskeletal and Neuronal Interactions 2004; 4(4): 428–435.
III. Limited Effects of Alimentary Zinc depletion on Rat Fracture Strength and Callus Formation. Ovesen J, Møller-Madsen B, Thomsen JS, and Andreassen TT. (Manuscript in preparation)

Abbreviations and Definitions

AMG	Histochemical autometallographic zinc technique
DEDTC	Diethyldithiocarbamate (chelator)
DRI	National Academy of Science on Dietary Reference Intakes
ρ	Bone density
E	Young's modulus (normalized stiffness)
ε	Strain (relative deformation)
F_{max}	Maximum load (force applied at fracture)
IGF-I	Insulin-like Growth Factor I
GH	Growth Hormone
MV	Matrix vesicles
Osteoblast	The metabolically active bone forming cell
Osteoclast	The cell capable of bone resorption
PTH	Parathyroid hormone
σ_{max}	Maximum stress (normalized maximum load)
TFIIIa	Protein transcription factor IIIa
TGF-β	Transforming growth factor-β
W_{abs}	Energy absorption
BMP	Bone morphogenic protein
Ihh	Indian hedgehog
PTHrP	Parathyroid hormone related peptide

Acknowledgements

This Ph.D. thesis is based upon studies conducted (during the period 2001–2004) at the Department of Orthopaedic Surgery, University Hospital of Aarhus, and the Departments of Neurobiology and Connective Tissue Biology, Institute of Anatomy, University of Aarhus. During this period I also finished my clinical education to become an orthopaedic specialist.

First of all, I want to thank my supervisors: Bjarne Møller-Madsen, M.D., D.M.Sc., who introduced me to the interesting research field of zinc and offered valuable inspiration and support.

I am greatly indebted to the late Lis Moskilde, M.D., D.M.Sc., who was generous with her time, inspiration, encouragement, and guided me expertly through the beginning of this thesis.

I am very thankful to Gorm Danscher, DVM.Sc., D.M.Sc., for his interest in my study and for his support.

I am very grateful to Jesper Skovhus Thomsen M.Sc., Ph.D., D.M.Sc. his collaboration and support on everything from methodological design to the writing process.

Troels Andreassen, M.D., is thanked for collaboration, support and for his analytical critical acuity.

The laboratory technicians Inger Vang Magnussen, Birthe Gylling-Jørgensen, Jytte Utoft, Dorete Jensen, and Herdis Krunderup are thanked for their excellent technical support.

Thorkild Alnor Nielsen was the experienced keeper of the animals and thaught me how to handle them.

Karin Wiedemann and Michael Hewitt are gratefully acknowledged for linguistic revision of the manuscript.

I would like to thank Erik Vittinghus, M.D. and his laboratory at Randers Hospital for valuable analyses and advise in determination of zinc concentration in bone.

I also wish to thank my colleagues at the Department of Orthopaedic Surgery; Aarhus, Aalborg, and Randers Hospital for their interesting discussions and many pleasant moments.

Above all, I am grateful to my husband, Søren Bo, for his love, patience, and support.

Contents

Chapter 1: Introduction

1.1. Clinical background

Zinc deficiency in human beings was first suspected in 1961 by Prasad et al.[91] They reported typical features of chronically zinc-deficient humans from Iran and Egypt, which were growth retardation, hypogonadism in males, poor appetite, mental lethargy, and skin changes. These pathological features were all corrected by zinc supplementation. In recent years similar cases have also been described in other parts of the world.[22, 84, 110] Zinc deficiency in humans occurs either when the amount of zinc in the food is low, or if it is not available for absorption, or if the demand for zinc is high, e.g. in relation to extreme sports activity,[70, 97] growing children, pregnancy, and lactation.[91] Furthermore, several authors have reported zinc supplementation to be effective for inducing growth in short children.[81, 84, 90] The significance of zinc has been extensively studied in different animals, and abnormal longitudinal bone growth and development have been found to be the most consistent characteristics of zinc deficiency.[18, 22, 32, 33, 44, 50, 61, 66, 73, 74, 83, 100, 108, 117, 119, 120]

In humans and animals, zinc deficiency and other different nutritional factors[80, 118] have been suggested to play an important role in the development of osteoporosis [10, 31, 43, 87, 96] and osteoporotic fractures.[35, 89] Although zinc has been proved to be essential for bone modeling and remodeling, only a few studies have focused on the effects of zinc on bone healing following a fracture.[48, 49, 71]

1.2. Diet and zinc

During recent years more attention has been given to the limited intake of zinc due to poor diets or eating habits.

Recommended daily allowances (RDA) for zinc were first established for human in 1970.[22] The parameters which were employed for establishing the specific human zinc requirement included: Plasma/serum concentration, hair zinc concentration, bone zinc concentration, response of growth and development of zinc to bone to zinc supplementation, and metabolic studies. 15 mg of dietary zinc per day were recommended for adults, 20 mg and 25 mg a day of zinc were recommended during pregnancy and lactation.[22]

Nearly every year new volumes are issued by the National Academy of Science on dietary reference intakes (DRI). These volumes provides recommended intakes, such as Recommended Dietary Allowances (RDA), for use in planning nutritionally adequate diet for individuals based on age and gender.[74] The updated recommendations are based on the Institute of Medicine's review of the scientific literature. Recommended dietary allowance (RDA) of zinc is still 15 mg/day for adults.[35, 82]

As mentioned above, the first cases of zinc deficiency in humans were reported by Prasad et al. in the Middle East.[91, 92] The unleavened bread, which is a major component of the diet in the Middle East, has rather high levels of phytates, chelating molecules that bind zinc and thereby decreases available zinc for absorption.[22, 91]

Another factor that has decreased the zinc intake is the change from iron- and zinc water pipes to copper or plastic pipes. This not only decreases the zinc intake, but the additional copper also hampers with the zinc absorption.

Zinc is found in a wide variety of foods. Good sources of zinc are seafood, red meat, poultry, vegetables, and nuts.[110] Zinc absorption in the digestive system is greater from a diet high in animal protein than from a diet

high in plant proteins. Several studies[22, 69, 70, 80, 81, 84, 110] in the Western world have shown suboptimal zinc intake and status in otherwise healthy humans – based on the recommendations by the National Academy of Science on Dietary Reference Intakes (DRIs).[82]

1.3. Biochemical properties of zinc

Zinc is a small atom (0.065 nm) and is present in biological systems as a divalent cation (Zn^{2+}). Very little zinc is found as free zinc ions as most zinc is bound primarily to proteins, with aspartate, glutamate, histidine, and cysteine being the major ligands.[111] The roles of zinc in enzymes, were decribed and classified by Vallee and Falchuk, as being catalytic, coactive, or structural.[111] The term catalytic denotes that zinc participates directly in enzyme catalysis, which means that if zinc is removed by chelating or other agents, the enzyme becomes inactive (e.g. carbonic anhydrase and alcohol dehydrogenase). Coactive zinc atoms regulate catalytic function in conjunction with another active metal ion in the same enzyme, but are not responsible for enzyme activity or stability. This is true for e.g. alkaline phosphatase (Zn and Mg) and phopholipase (Zn and Zn). Structural zinc ions provide structural stability to certain proteins, e.g. alcohol dehydrogenase and aspartate transcarbamylase.[111]

In recent years, the role of zinc ions as a key structural component of a large number of proteins has been investigated. Berg and Yigong reported that the ability of zinc to bind specifically on a range of tetrahedral sites in macromolecules appears to be responsible for the wide range of zinc-stabilized structural domains (e.g. TFIIIa).[17]

1.4. Normal longitudinal bone growth

The longitudinal bone growth is related to the epiphyseal growth plate. The epiphyseal growth plate is a cartilaginous structure located between the epiphysis and the metaphysis. This cartilaginous template consists of proliferating chondrocytes organized in columns in a pattern according to their stage of maturation (Figure 1).[42, 86] In growing individuals, this calcified cartilage serves as a template for bone formation (i.e. endochondral ossification). This endochondral ossification involves undifferentiated mesenchymal cells forming a cartilaginous model by continuos proliferation and maturation of chondrocytes. Osteoclasts then invade the calcified areas of cartilage followed by osteoblasts, which then lay down ostoid. In this way the cartilaginous model is replaced by woven bone as length increases. The woven bone has poorly mechanical properties and must be remodeled into lamellar bone, which is the type of bone tissue normally found in cortical bone in the diaphysis and in cancellous bone in the metaphysis and epiphysis. This remodeling process involves a tightly coordination between the bone resorbing osteoclasts and the bone forming osteoblasts regulated by cytokines, hormones, and signal-

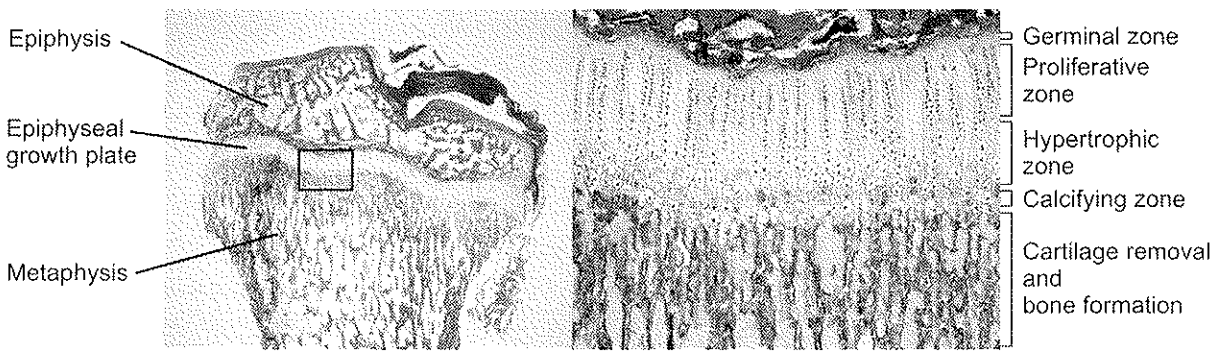

Figure 1. The proximal tibial epiphyseal growth plate and its cellular organization in the rat. The box in the left image indicates the boundary of the right image.

ling receptors.[64]

This endochondral calcification process is closely related to that seen in fracture healing. The fracture healing response can be divided into 3 phases: 1) Inflammatory, 2) repair, and 3) remodeling.[95, 67] In the inflammatory phase haematoma organizes and is invaded by fibrovascular tissue. This fibrous tissue is mineralized to form primary callous of woven bone through endochondral bone formation. Maturation of the callus (woven bone) occurs during the remodeling phase. In this last stage of fracture repair the immature woven bone is resorbed by osteoclasts and replaced with lamellar bone by osteoblasts.[64, 95]

It has been suggested that matrix vesicles in the hypertrophic zone are involved in the induction of calcification of the growth plate.[3, 45, 88] According to Anderson, the matrix vesicle's biogenesis in the growth plate is linked to the chondrocyte cell cycle. They arise in the skeletal tissues by budding and then pinching-off from the outer cell membrane of the chondrocytes and osteoblasts.[3] He described the mechanism as a biphasic event: Phase 1 is initiated by cells generating calcifiable matrix vesicles and releasing them into sites of intended calcification. Phase 2 begins with breakdown of matrix vesicle membranes, exposing hydroxyapatite to the extracellular fluid, after which mineral crystal proliferation is regulated by extracellular conditions. This hypothesis has been supported by other investigators.[45, 46, 47, 56, 99] Thus, electron microscopically matrix vesicles are extracellular 30–200 nm-diameter membrane-invested particles within the matrix of bone and cartilage. Within the single covering membrane of matrix vesicles lies the vesicle sap, which is often electron dense because it contains needle like crystals of hydroxyapatite mineral.[3]

The normal bone growth (endochondral ossification) is regulated by a number of systemic and local growth factors, such as growth hormone (GH), insulin-like growth factors (IGF-I), parathyroid hormone (PTH), sex hormones, and transforming growth factor β (TGF-β).

Growth hormone is shown to have two effects on the epifyseal growth plate; 1) a direct stimulatory effect on the precursor cells and 2) an indirect effect mediated through the production of IGF-I.[6, 11, 20, 86]

Insulin-like growth factor I is also found to regulate proliferation and differentiation of cartilage in the growth plate.[11, 86] However, the effect of IGF-I differs from that of GH in that IGF-I stimulates the chondrocytes in the proliferative cell layer of the growth plate and not the precursor cells as seen with GH.[11]

Parathyroid hormone is involved in the regulation of both bone resorption and bone formation.[78] Several studies have shown PTH, when administered intermittently, to be a bone-specific anabolic agent inducing effect at both the endosteal and periosteal surfaces of the bone.[4, 5, 6, 79, 107] Normal development of the growth plate requires coordination of proliferation and differentiation of the chondrocytes and osteoblasts. A series of genetic studies have shown that the proliferation in the growth plate is under control of a local feedback loop that primarily involves two signaling molecules synthesized by growth plate chondrocytes: PTHrP (parathyroid hormone related peptide) and Indian hedgehog (Ihh). Indian hedgehog (Ihh) produced by prehypertrophic and hypertrophic chondrocytes, stimulates chondrocyte differentiation and the production of PTHrP, which negatively regulates chondrocyt differentiation.[57, 60, 65, 112] Kobayashi et al.suggested that Ihh positively controls differentiation of periarticular chondrocytes independently of PTHrP. Thus, concluding that PTHrP and Ihh controls differentiation of the growth plate chondrocytes at multiple steps.

The sex hormones act primarily through regulation of GH secretion, but some studies have demonstrated that sex hormones also have direct effects on growth,[11, 87] independent of GH secretion.

Transforming growth factor-β is isolated from bone matrix of calcified cartilage and bone, but the main role of TGF-β has not been

determined.[11, 87] In vitro studies have shown that a large family of hormones is related to TGF-β. These hormones are generally classified as bone morphogenic proteins (BMPs) and are known to be osteoinductive. Osteoinduction is a process by which a stimulus induces undetermined osteoprognitor stem cells to enter osteoblastic differentiation leading to mature osteoblasts.[64, 95] It has also been suggested that TGF-β acts on the perichodrial and periarticular cells to increase PTHrP synthesis.[15]

The molecular mechanisms that regulate osteoblast differentiation and bone formation have resulted in several genetic studies. Runx2 (Cbfa/AML) has been identified as a member of the transscription factors essential for osteoblast differentiation and bone formation.[58, 62]

Longitudinal bone growth is regulated not only by the factors mentioned, but also by e.g. nutrition status (i.e. suboptimal zinc intake), severe diseases, and genetic potential.

Table 1. Prior investigations of the effect of zinc on bone growth.

Authors	N	Species	Sex/age	Zinc dose in diet (mg/kg)	Treatment period	Method of evaluation	Effect
Todd 1934	–	Rats	Male & female 3 weeks	5	15 weeks	B C	Stimulation
Forbes 1958	103	Rats	Male Weanling	7/18/31/52	6 weeks	B T	Stimulation
Becker 1966	192	Rats	Male	20 ± vitamin D ± vitamin A	3 weeks	B T	Stimulation Stimulation No effect
Prasad 1967	12	Rats	Male Weanling	10/55	6 weeks	B F K Bi	Stimulation
Swenerton 1968	164	Rats	Male & female Weanling	1/4/60/100	12 weeks	B K Bi C	Stimulation
Bergmann 1969	20	Rats	Female 3 weeks	73	18 days	B T K P F	Stimulation
Williams 1970	96	Rats	Male & female Weanling	3/6/9/20/112	35 days	B K F	Stimulation
Prasad 1971	12	Rats	Male Weanling	110	5 weeks	B K Bi	Stimulation
Fernandez 1973	108	Rats	Male Weanling	125	4 weeks + 6–10 days	B K Bi	Stimulation
Wing 1975	48	Rats	Female 3 weeks	-		T	-
Weigand 1978	36	Rats	Male Weanling	5.6/10.6/18.2/ 39/70/141	15 days	B T	Stimulation
Milachowski 1980	60	Rats	Male	± zinc inj.	8 weeks	B K P R H	Limited effect
Koo 1980	–	Rachitic chicks	? 1 day	100 ± vitamin D	3 weeks	B T	Stimulation No effect
Walwork 1981	45	Rats	Male Weanling	?	30 days	B F Bi P	Stimulation
Suwarnasarn 1982	20	Rats	Male 16–22 days	25	25 days	K P M H Bi	Stimulation
Leek 1984	29	Monkeys	Female & male	100	12 months	R	Stimulation

Yamaguchi 1986	–	Rats	Male Weanling	1/100 per 100 g body wt.	4 days	B Bi	Stimulation
Dørup 1991	17	Rats	Female 3–4 weeks	30/100/500 µmol	50 days	B Bi P	Stimulation
Browning 1997	76	Rats	Male 5–6 weeks	100 ±IGF-I ±Megestrol	8 days	B F	Stimulation No effect B F No effect on B
Ninh 1998	36	Rats	Female	75 ± IGF-I	4 weeks	B F Bi	Stimulation No effect
Igarishi 1998	–	Rats	Male 4 weeks	3–10 per 100 g body wt.	28 days	Bi Fr P K	Stimulation
Seco 1998	85	Rats	Female 93 days	85/102 ± exercise	11 weeks	B Hi	Stimulation
Eberle 1999	24	Rats	Male 6 weeks	60	42 days	B P K H Hi Bi	Stimulation
Ma 2000	–	Rats	Female & male Weanling	1–2 per 100 g body wt.	1 & 35 days	K Bi	Stimulation

Methods of evaluation: M) Mechanical testing; H) Histological examination; C) Clinical examination; R) Radiographic examination; B) Bodyweight; Fr) Fracture; K) Concentration in bone; P) Concentration in plasma; Hi) Histomorphometry; T) Turnover parameters; F) Food intake; and Bi) Biochemical examination.

1.5. The effects of zinc deficiency on longitudinal bone growth

A number of investigators have during the last seventy years shown results concerning the influence of zinc deficiency on bone growth (Table 1).

As early as in 1934 Todd et al. observed a beneficial effect on the growth of rats upon the addition of zinc to the diet.[108] In 1958 Forbes and Yohe reported a series of investigations to elucidate the zinc requirement of the rat and the excretory pattern of dietary zinc by observing the anabolic effect of zinc on weight gains.[41] Becker and Hoekstra (1966) studied the effect of dietary vitamin D on the absorption, distribution and turnover of zinc in growing rats and reported an increase in uptake of zinc by skeletal tissue and an increase in skeleton weight when treated with vitamin D.[16]

Swenerton and Hurley (1968) described the signs of severe zinc deficiency in rats as extreme retardation of growth, immature hair, and dermal lesions. They also reported reduced concentrations of zinc in the femurs of the zinc-deficient rats.[104] Bergman (1969) observed reduced rate of growth and significantly lower zinc concentrations in spongy bone samples in rats fed a zinc-deficient diet. No significant differences were reported in zinc concentration for compact bone.[18] Williams and Mills (1970) reported results obtained in both male and female rats, that within the range of 6 -12 mg zinc/kg diet, there was a close relationship between rate of weight and dietary Zn concentration.[116]

Prasad et al. (1967 and 1971) reported that zinc deficiency in weanling rats resulted in growth retardation and reduced activities of certain enzymes in testis, bones, esophagus, and kidneys.[92, 94] Fernadez-Madrid et al. (1973) also reported decreased gain in weight of rats fed a zinc-deficient diet, and by comparing rats with pair-fed and ad libitum fed controls they demonstrated, the impairment in protein and collagen synthesis observed in zinc-depleted rats was due to deficiency and not to differences in caloric intake.[39] Wing (1975) and Weigand (1978) investigated the turnover of Zn in rats. Weigand and Kirchgesser reported that rats attempt to control zinc balance homeostatically according to needs by regulation the extent of intestinal absorption of dietary Zn and the rate of fecal excretion of endogenous Zn.[115]

Milachowski et al. (1980) reported a sig-

nificant decrease of bone-zinc concentration after fracture that could be balanced by zinc administration.[71]

Koo et al. (1980) investigated the effects of vitamin D on the absorption of zinc in zinc-deficient and zinc-adequate chicks, and found that vitamin D was not a part of the zinc homeostatic mechanism, but the body weights and bone-zinc concentrations of the zinc-depleted chicks were significantly lower than those receiving zinc-supplemented diet.[59]

Suwarnasarn et al. (1982) reported narrowed epiphyseal plates and less force required to displace the epiphysis in zinc-deficient rats. They also reported significant decrease in bone-zinc concentration in zinc-deficient rats.[103]

Leek and al. (1984) reported fetal and infant skeletal effects in rhesus monkeys and found radiographic features similar to human rachitis syndromes (absence of epiphyseal ossification centers and widened growth plates).[61]

Yamaguchi and Yamaguchi (1986) investigated the effects of zinc on enzymes of femoral tissue in weanling rats. They found increased concentration of alkaline phosphatase related to DNA synthesis of the femoral.[120]

Some studies have shown that animals with zinc deficiency had lower serum concentrations of IGF-I and therefore retarded growth. However, if the serum concentrations of IGF-I was restored by IGF-I infusion to the zinc-depleted rats, as shown by Dørup et al. (1991), this did not reverse growth retardation.[32] Browning et al. (1997)[20] and Ninh (1998)[83] reported similar results in growing rats.

Igarishi and Yamaguchi (1999) reported that administration of zinc caused a significant increase in calcium content, alkaline and acid phosphatases, protein and DNA contents in the femoral-diaphyseal tissues of rats with fracture healing. Femoral mineral density and bone-zinc concentration were also increased by zinc-supplementation.[49]

Seco and al. (1998) reported that rats exposed to strenuous exercise showed low femoral longitudinal development and significant axial and peripheral osteopenia and the effectiveness of zinc supplementation in preventing this osteopenia.[100]

Eberle and al. (1999) reported reduced growth in zinc deficient rats by significantly lower body weights, plasma, and femur zinc concentration. They also did a histomorphometric evaluation of the distal femoral metaphysis and showed that zinc deficiency led to a reduction in cancellous bone mass, osteopenia, and to a deterioration of trabecular bone architecture, with fewer and thinner trabeculae.[33]

Ma and Yamaguchi (2000) investigated the alteration in bone components with increasing age of newborn rats to determine the role of zinc in the development of bone growth and found that bone alkaline phosphatase activity, calcium, DNA, and zinc content significantly increased with increasing age and with oral zinc supplementation.[66]

Matrix vesicles (MV), being implicated in the initiation of calcification, have resulted in several in vitro studies testing the effects of zinc on calcium uptake in matrix vesicles. Sauer et al. isolated MV from chicken growth plates and found that zinc ions act as an endogenous regulator of MV Ca^{2+} uptake.[99] Likewise, Hsu and Anderson also found that zinc supplementation to matrix vesicles isolated from the epiphyseal cartilage of rachitic rats enhanced calcium uptake.[46] This hypothesis has been supported by other studies.[23, 56, 63] Rodríguez and Rosselot suggested that the effect of zinc on linear growth might be explained by an increase in the proliferation rate of the proliferating chondrocytes and an increased synthesis of highly charged proteoglycan molecules which decreased mineralization.[98]

Another described alternative mechanism of zinc deficiency is that zinc indirectly affects cellular activities in the growth plate through changing levels of hormones or growth factors. As mentioned above Ninh et al. suggested that growth retardation induced

by zinc deficiency is associated with both low serum IGF-I concentrations and inhibition of the anabolic action of IGF-I.[83] Dørup et al. likewise suggested that growth retardation in zinc-deficient rats might be mediated through reduced serum IGF-I production.[32]

Previously studies suggest that vitamin D play a role in the metabolism of bone regulation of zinc ions.[7, 16, 38, 59]

Thus, the results during the last seventy years of investigations of the effect of zinc administration on bone growth are rather heterogenous and primarily limited to reports of weight gain, bone-zinc concentration, whereas only a few histological, biomechanical, and histomorphometrical studies have been reported. In summary, zinc deficiency induces changes in the growth plate that lead to retarded longitudinal bone growth. Therefore, it is clear that no previously published studies have investigated the effects of alimentary zinc on bone strength and bone strength of healing fractures. In addition, only a few studies have examinated the localization of zinc ions in growing bone and cartilage.

1.6. Aims

The overall purpose of this Ph.D. thesis was to assess the skeletal effects of alimentary zinc depletion and supplementation on bone growth, modeling, and remodeling. Accordingly, the study was divided into three main experiments:

- Investigation of the bone quality after alimentary zinc depletion and supplementation in an animal model of intact, growing rats. A biomechanical study. (Paper I).
- A histological description of bone changes and the amount and localization of zinc ions following alimentary zinc depletion and supplementation in an animal model of intact growing rats. A histological study. (Paper II).
- The effects of alimentary zinc depletion and supplementation on fracture healing in an animal model with a standardized closed fracture. A biomechanical and histological study. (Paper III).

Chapter 2: Material and Methodological Considerations

2.1. Animals and animal care

Fourty five 4-weeks-old male Wistar rats were used for the investigations, except for the fracture study, where eighty 12-weeks-old male rats were used (I, II, III). The animals were delivered by Møllegaard's Breeding Center, Ltd., Ejby, Denmark. The rats 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 chosen for several reasons:

- It is a well-known laboratory animal used in numerous bone studies.
- It is a homogeneous population, which is readily available.
- The laboratory staff has great experience in handling rats.
- The fracture and fixation model in rats is well known in the laboratory and the procedure is easily tolerated by the animals, resulting in relatively low animal suffering. [11, 12, 13, 14]

However, several problems are associated with the use of a rat model, due to the differences between human and animal bone reaction and morphology. It is often believed that rats grow continuously throughout life, because laboratory animals fed ad libitum continue to increase their body weight for a substantial part of their lifespan.[11] However, several studies indicate that this is not true. The growth of the rat is rapid until 170 days and thereafter it declines markedly.[53] In old rats there is no longer evidence of osteogenesis in the growth plate.[54] Furthermore, rats do not have the same pattern of remodeling as humans, as they lack Haversian oteons, which precludes intracortical remodeling. However, intracortical remodeling can be seen after a period of time. This, corroborated by the fact that rats have cancellous bone remodeling,[37] makes it likely that the basic mechanisms of bone turnover in humans also exist in the rats. [52, 53, 54]

2.1.1 *Animal sacrifice*

The animal used for biomechanical testing (Paper I) were sacrificed after 4 weeks by decapitation under anesthesia with mebumal (50 mg/ml). Both hindlimbs were dissected from the body and all extraneous tissue was removed. The bones were sealed in plastic wrap and immediately frozen at –20° C.

The animals used for histological evaluations were sacrified after 4 weeks (Papers I and II), or after 21 days and 56 days (Paper III). They were anesthetized with mebumal 50 mg/ml and transcardially perfused for 10 minutes with 0.5% sodium sulphide solution, followed by perfusion with 3% gluteraldehyde in 0.1 M phosphate solution for 3 minutes. Both hindlimbs were dissected from the body and postfixed for 1–4 hours in the gluteraldehyde fixative.

The animals used for biomechanical testing of the fracture healing (Paper III) were sacrified after either 21 days or after 56 days by an intra-peritoneal injection with mebumal (150 mg/g body weight). The tibia were dissected from the body and kept in buffered Ringer's solution at 4°C until mechanical testing, which was performed within 6 hours.

2.2. Diets

All animals received a semisynthetic diet (Altromin C1040 (special recommended diet for laboratory rodents, Altromin Gesellschaft für Tierernaehrung GmbH, Lage, Germany [Import: Brogården, Gentofte, Denmark]) with differing amounts of zinc added. All diets contained calcium 9508 mg/kg, phosphor

7540 mg/kg, and vitamin D3 500.000 IE/kg. The metabolizable energy of the diet was 3564.46 kcal/kg. The composition of the diet fed to the rats is given in Table 2.

Table 2. Composition of the Altromin C1040 diet.

Ingredient	g/kg diet
Protein	179.533
Fat	50.318
Fiber	39.756
Water	67.436
Ash	52.901
Disaccharides	307.674
Polysaccharides	293.176
Variable ingredients (incl. zinc)	9.206

As the recommended daily allowances (RDA) of zinc for humans[82], also a dietary requirements for rats exists and are recommended by the Institute of Laboratory Animal Research (ILAR). These recommendations are also based on review of the scientific literature reporting metabolic studies of zinc. But the literature reveals many discrepancies in the level of zinc found to be sufficient for rats. Forbes and Yohe reported that the zinc requirements for rats is 18 ppm (= 18 mg zinc/kg diet) in a diet containing isolated soybean protein.[41] In contrast, Swenerton and Hurley reported a requirement of zinc of 100 ppm (>40 ppm for female and >60 ppm for males).[104] Williams and Mills stated that 5–13 ppm was sufficient,[116] Prasad and Oberleas recommended 110 ppm,[92] and Fernandez-Madrid et al.125 ppm.[39]

Zinc is often considered to be relatively non-toxic both in humans and animals, but cases of toxicity in excess of 5000mg/kg diet have resulted in reduced growth, anorexia, anemia, and death.[51]

According to the Subcommittee on Laboratory Animal Nutrition (LAN) 1995, dietary zinc requirements for weanling and adult rats are 12 mg zinc/kg diet and for lactating rats 25 mg zinc/kg diet.[51]

The Subcommittee on Laboratory Animal Nutrition (LAN) reported that several investigators often use control diets that contain 100 mg zinc/kg diet. However, LAN observe that although this amount of zinc does not represent a "toxic" risk to rats, it might be more accurate to classify a 100 mg zinc/kg diet as a zinc supplemented diet instead of a control diet.[51]

According to the LAN recommendations we planned study I and II to be dose-response studies and we chose diets with 2 mg (hypo), 47 mg (control), and 60 mg (hyper) zinc/kg. Using the experience obtained in the first two studies we chose to conduct the fracture study with only two different zinc contents rather than several different doses. As we suspected the effects of the alimentary zinc diets to be somewhat weaker in the fracture study we also chose to elevate the zinc supplemented dose to 100 mg/kg diet.

2.3. Zinc contamination

It is impossible to abolish zinc from the environment, but we tried to eliminate sources of zinc contamination by housing the rats in metal-free cages, using distilled water, and plastic equipment for storage of the dietary ingredients. However, contamination cannot be completely eliminated. Bergmann reported that zinc intake due to coprophagy and ingested hair from other animals could be a possible source of contamination.[18]

Since the intramedullary nail (Kirschner wire) used in the fracture model was introduced to the marrow cavity, this could be a potential source of zinc supply/contamination. Therefore, we analyzed the drain of zinc from the Kirschner wire in the following way: A Kirschner wire was placed into a test tube with 25 ml physiological NaCl and stored in a heating cupboard at 37°C for 56 days, after which the amount of zinc in the sample was measured by atomic absorption spectrophotometry (AAnalyst 100, Perkin-Elmer, Germany). A control test with physiological NaCl in the tube without a Kirschner wire was also

performed.

The results of this investigation are shown in Table 3.

It can be seen from Table 3 that the average zinc content is significantly higher in the samples containing a Kirschner wire than in the control samples. Consequently, it cannot be excluded that the zinc-deficient rats had some zinc supply from the Kirschner wire. However, this source of contamination provided only minimal amounts of zinc. The discharge of zinc from each wire was approximately 0.5 µg during the 8 week observation period (Kirschner wire: 0.51 ± 0.06 µg, vehicle without wire: 0.16 ± 0.03 µg, (mean ± SD, $p = 0.008$)).

Table 3. The release of zinc (mean ± SD).

	NaCl + K-wire (µmol zinc/L)	NaCl (µmol zinc/L)
1	0.357	0.087
2	0.234	0.132
3	0.337	0.064
Mean	0.309[a] ± 0.066	0.094 ± 0.034

Key: [a]Significantly different from NaCl without K-wire.

According to Williams and Mills the total amount of zinc in a weanling rat giving a diet with 12 mg zinc/kg is approximately 3.6 mg zinc.[116] One third of the total amount of zinc is located in the bone[7], which gives a total amount of approximately 1.2 mg zinc in the skeleton of a growing rat. Comparing the total amount of zinc in the skeleton from a growing rat and the amount of zinc given through the diet in our study (zinc-deficient group(2 mg zinc/kg diet): Approximately 2.5 mg during 8 weeks of observation, zinc-sufficient group (100 mg zinc/kg diet): Approximately 125 mg during the 8 weeks of observation) with the possible contamination from the Kirshner-wire (0.5 mg during the 8 weeks observation period), this is a very little but still measurable source of zinc contamination.

2.4. Concentration of zinc in bone

Zinc concentrations in tissue can be determined exactly by atomic absorption spectrophotometry, as described by several authors.[8, 33, 49, 71, 117] Using this method it is possible to determine quickly and quantetively the amount of zinc in the range of a few micrograms per gram or millilitre of tissue. But the material to be tested has to be liquefied. Therefore bone samples were carefully dissected from soft tissue and ashed for 48 hours at 580°C. When they had cooled to room temperature, the ash weights were recorded and the ash dissolved in 1.2M HCL (1:3). The zinc concentration was determined using an atomic absorption spectrophotometer (Model AAnalyst 100, Perkin-Elmer, Germany). In order to determine the accuracy of the method, a series of aqueous controls were carried through the investigations and coefficients of variations were 0.03 for these controls. These results prove that the proposed method itself can give acceptable results in spite of the several additions and manipulations involved. Zinc content was expressed as µg of zinc per g dry bone tissue.

2.5. The fracture model

Several fracture models have been described for use in rats. Femoral or tibiofibular fractures are the most commonly used in the rat model. This procedure has the advantage that the healing fractures can be compared with the contralateral non-fractured leg. In the present investigations we used the closed medullary nailing as described by Bak[11] and Bak and Jensen.[14] The animals were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ, USA).

A unilateral standardized closed tibial fracture was produced by three-point bending above the right tibiofibular junction with a specially designed, adjustable forceps. Closed medullary nailing was performed with a Kirschner wire under sterile conditions. Identical Kirschner wires (0.9 mm diameter) were used for all animals. Postoperatively, contact X-rays were captured to secure correct localization of the fracture and the intramedullary nail. Unprotected weight-bearing was al-

lowed, and the animals resumed normal activity after recovery from the anaesthesia.

2.6. External callus volume and dimensions

In the fractured tibiae, the external medial-lateral and anterior-posterior diameters of the callus were measured at the fracture line by a digital sliding calliper. At the corresponding level of the non-fractured tibia the transverse and antero-posterior diameters were measured. Total volumes of both fractured and contralateral intact tibiae were gauged using Archimedes' principle, and the external callus volume was calculated as the volume of the fractured tibia minus the volume of the contralateral intact tibia.[13]

Several problems exist in these measurements, because of the inhomogeneous nature of the fracture callus in size and geometry. For the measurement of callus dimensions of such healing fractures Bue Bak reported coefficients of variations to be 0.02.[11] In this study we compared the right-sided fractured tibia with the left-sided non-fractured tibia as a reference, as Bak and Jensen have shown that the right and left tibia of the rat is symmetrical.[14]

2.7. Biomechanical testing

The mechanical properties of bone can be measured in the following biomechanical test procedures: Compression, tension, torsion, and bending (three or four point) tests. Which test to choose depends on 1) the kind of bone being tested, 2) the age of the bone and the anatomical location, and 3) variations in the testing conditions. Concerning fracture healing, torsional or bending tests seem to be the most appropriate.[21, 34, 109] In the fracture study the bending test was used, because this test is useful for measuring the mechanical properties in smaller bones from rodents. However, to describe the properties of bone specimens, especially cancellous bone, the compression test is a popular technique.[75, 78, 105] Therefore, in addition to the three-point bending the compression test of the trabecular bone-rich distal metaphysic was also performed in Paper I. The compression test was used, knowing that it is less accurate than tensile tests due to end effects on specimens during testing.[77, 85]

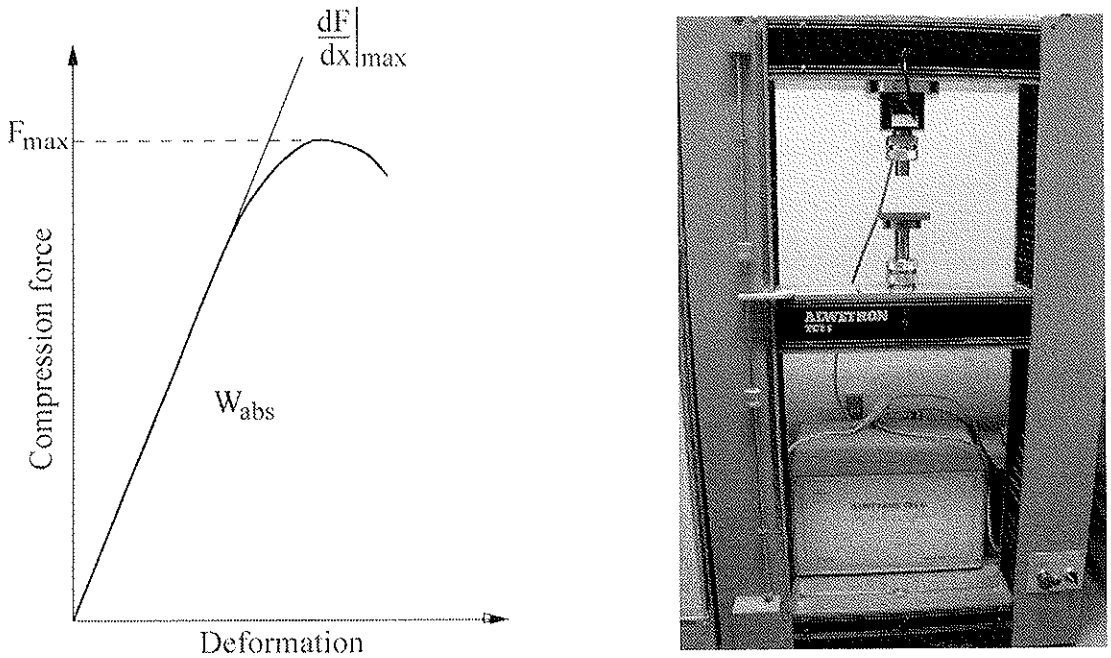

Figure 2. Left: Load-deformation curve. Right: Alwetron materials testing machine.

Keaveny et al. reported systematic and random errors of about 12.5% associated with end-artifacts in the compression test for trabecular bone, but the same methods were used in these studies and therefore the results should be comparable.[55]

We did not evaluate the reproducibility of the procedures used for the mechanical testing, but Bak and Jensen evaluated the reproducibility of the same three-point bending procedure as used in our study by testing identical K-wires. [14] In this way they found that coefficients of variations were 0.01 for maximum load and maximum stress, 0.03 for maximum stiffness, and 0.11 for energy absorbtion.[11, 14]

The mechanical testing was performed on a materials-testing machine (Alwetron, TCT5; Lorentzen and Wettre, Stockholm, Sweden). Load-deformation curves (Figure 2) were recorded, and analyzed by personal computer (Prolinea 4/33; Compaq, Houston, TX, USA).

On the day of testing, the right femora were slowly thawed at room temperature. The specimens were placed in Ringer's solution for 1 hour before the following biomechanical tests were conducted.

2.7.1. Three-point bending test of the femoral diaphysis

The length of the femora was measured with an electronic calliper, and the midpoint was marked. The femora were placed in a testing jig constructed for three-point bending tests (Figure 3).

The distance between the supporting rods had fixed length of L = 15.73 mm. Load was applied at a constant deformation rate of 2 mm/min with a rod at the midpoint of the femur. Load-deformations curves were recorded and the following parameters calculated according to Turner and Burr:[109]

F_{max} The maximum load is found as the maximum point on the load-deformation curve (N).

d The deformation at maximum load (m).

E Young's modulus is found as the maximum stress divided by the strain (Pa).

σ_{max} The maximum stress is found as the maximum load times the length between the supporting rods times the length from the bending axis to the upper bone surface divided by 4 times the axial moment of inertia around the bending axis (Pa).

ε The strain is found as 12 times the length from the bending axis to the upper surface times the deformation at maximum load divided by length between the supporting rods squared.

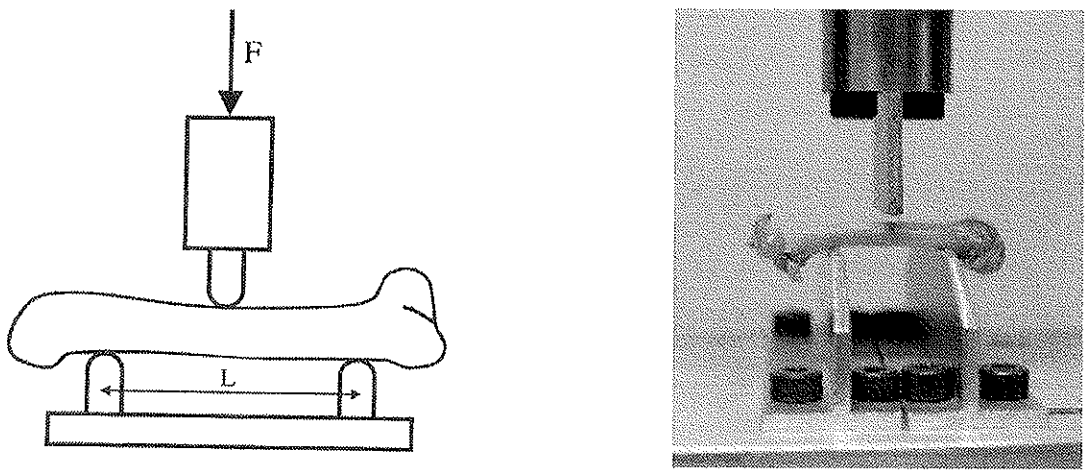

Figure 3. Left: Schematic drawing of the 3-point bending testing jig. Right: The testing jig used.

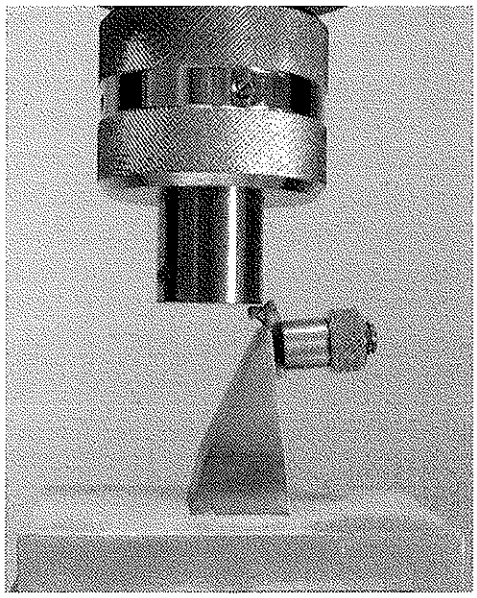

Figure 4. Device for fixation of the proximal femoral metaphysis.

2.7.2. Biomechanical testing of the femoral neck

The proximal femora were mounted in a device for standardized fixation to produce a cervical, extracapsular fracture of the femoral neck. A vertical load conducted by a metal cylinder was applied to the top of the femoral head with a constant rate of 2 mm/min until fracture of the femoral neck (Figure 4).

Load-deformation curves were obtained and the following parameter determined:

F_{max} The maximum load on the load-deformation curve (N).

2.7.3. Biomechanical testing of the femoral metaphysis

From each femora an approximately 3.9 mm-thick section with planoparallel ends was sawed from the distal part of the metaphysis just above the anterior uppermost part of the patellofemoral joint cartilage. The sections were sawed with a diamond precision-parallel saw (Exact; Apparatebau, Otto Herman, Nordstedt, Germany). The volume of the specimens was estimated by weighing the specimens before and during immersion in water with an electronic balance (Mettler AG245, Mettler-Toledo, Nänikon-Greifensee, Switzerland) equipped to measure volumes. The length of the specimens was measured by use of a micrometer and the average cross-sectional area was calculated by dividing the bone volume with the specimen height. The sections were tested along the proximal-distal axis with a constant deformation rate of 2 mm/min. After testing the specimens were ashed (105°C for 2 hours and 580°C for 24 hours). The following parameters were determined:

F_{max} The maximum load is found as the maximum point on the load-deformation curve (N).

W_{abs} The energy absorption is the amount of energy absorbed in the bone specimen during compression until the failure point. It is found as the area under the load-deformation curve until the failure point (J).

σ_{max} The maximum stress is found as the maximum load divided by the average cross-sectional area (Pa).

E Young's modulus is found as the maximum slope of the load-deformation curve dF/dx, found by linear regression in a local vicinity of the point with maximum slope, multiplied by the uncompressed specimen length and divided by the cross-

sectional area of the specimen.

ρ The apparent bone density is found as the ash weight of the specimen divided by the volume of the specimen (kg/m^3).

2.8. Static histomorphometry

A 200-µm-thick section was sawed from the proximal part of the femoral diaphysis as close as possible to the fracture point from the three-point bending. The section was placed in a stereomicroscope (SZ-40; Olympus, Tokyo, Japan) and a CCD video camera (WV-CD 130; Panasonic, Osaka, Japan) was attached to the microscope and connected to a PC (Prolinea 4/33; Compaq, Houston, TX, USA) equipped with a frame-grabber card (LifeView; Anamation Technologies, Inc., Taipei, Taiwan). Images of the sections were captured and transformed into black-and-white images by threshold filtering. An image of a reference was captured at the same magnification in order to enable conversion of pixel coordinates to physical coordinates. With an in-house-developed software, the bone area, marrow area, tissue area, axial moment of inertia, and the distance from the bending axis to the upper surface of the bone were measured using a computerized method (Figure 5).

2.9. Histology. AMG and Goldner Trichrome

Zinc in biological tissues is known to be found in two pools.[28] One pool is firmly bound to proteins, where zinc is involved in maintaining the three-dimensional form of the molecules.[17] The other pool of zinc is present as loosely-bound or free zinc ions, often stored in secretory vesicles. Such vesicular pools of zinc ions have been described from many different secretory glands and from the nervous system.[26, 27, 29, 30, 36, 40, 101, 102, 121] Recently, the autometallographic (AMG)[97] zinc technique has been used to trace zinc ions in bone.[28]

There exist two AMG approaches for tracing zinc ions in tissues: The in vivo selenium $ZnSe^{AMG}$ technique, and the in vitro sulphide or immersion ZnS^{AMG} techniques. They relate in situ capturing of zinc ions in zinc-selenium or zinc-sulphur nanocrystals that are subsequently silver enhanced by AMG development of the tissue sections.

Longitudinal, 200-µm-thick transdiaphyseal sections 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 min.

2.9.1. AMG development

The details of this AMG method have been described by Danscher and al.[28]

- *Protective colloid*: Dissolve 2 kg crude gum arabic resin drops in 4 l deionized water, stir intermittently for 5 days and then filter through several

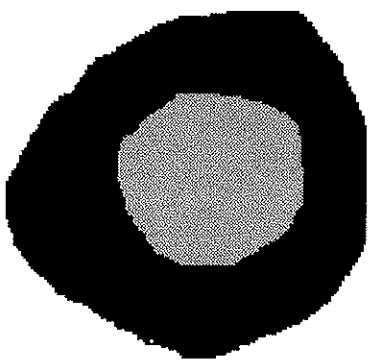

Figure 5. Femoral cross section showing bone area (black), marrow area (grey), and tissue area (black+grey).

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 extraneous light during the 60–90 min 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 and counterstained with toluidine blue.

2.9.2. Controls

To ensure that the AMG staining had been caused by zinc ions, controls were performed blocking the zinc ion pools in vivo with the low toxic chelator diethyldithiocarbamate (DEDTC). As controls for the specificity of the AMG method in these studies, eight animals were used (four animals in Paper II and four animals in Paper III). 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.

2.9.3. Goldner Trichrome

Some sections that had not been AMG developed were stained with Goldner Trichrome in order to measure the thickness of the growth plate, and to perform a histological description.

The 10-µm-thick Goldner Trichrome stained femoral 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 ×100. 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 oriented parallel to the long axis of the femora.

2.10. Statistical analysis

All statistics were performed using SPSS 10.0 (SPSS INC. Chicago, Illinois USA). In all the statistical tests $p < 0.05$ was considered significant.

Kruskal-Wallis test (Kruskal-Wallis one-way analysis of variance)[24] which is a non-parametric test was chosen in case the data were not normally distributed.

One-way analysis of variance (ANOVA)[9, 19] was used to compare the data from the three groups (2 mg, 47 mg, and 60 mg). Before carrying out the ANOVA, the data were checked for normality using a Q-Q plot (if the sample is from a normal distribution, points will cluster around a straight line). In the event of a significant outcome of the ANOVA test, a Bonferonni test or a *t*-test was used as post hoc analysis. The data are presented as mean value ± the standard deviation (SD), unless otherwise stated.

2.11. Ethical considerations

The human trial (study) was approved by the Medical Ethical Committees of Aarhus County, J.nr. 20010174 and was conducted in accordance with the recommendations of the Helsinki Declaration II. Prior to inclusion,

patients and their parents gave their informed written consent. The animal experiments complied with the Danish Animal Experiment Inspectorate and were approved by the Danish Ministry of Justice.

Chapter 3: Summery of Results and Discussion

3.1. Animal body weight

Papers I and II showed that the mean body weight of the young animals (4 weeks old) was significantly lower in the zinc-depleted group and significantly higher in the zinc-supplemented group than in the normal group, thus exhibiting a dose-dependent response (Table 4).

Neither after 21 days of healing nor after 56 days of healing was any difference in body weight gain found between zinc deficient and zinc sufficient animals (Table 5).

3.2. Femoral length

Papers I and II showed that mean femoral length of the young animals (4 weeks) old was significantly lower in the zinc-depleted group and significant higher in the zinc-supplemented group than in the normal zinc group, thus again exhibiting a dose-dependent response (Table 6 and Figure 6).

From the above results we believe that zinc supplementation can improve rates of growth in weaning rats; the body weights and the lengths of the femora were higher in the zinc-supplemented rats. Because the rats in our study had free access to the diets, it may be argued, as it has been in several studies,[1, 33, 113] that the skeletal effects were not due to zinc depletion but due to reduced food intake. We cannot totally exclude this possibility. However, as Fernandez-Madrid et al. demonstrated, by comparing zinc-deficient rats with pair-fed and ad libitum-fed controls, the impairment in protein and collagen synthesis observed in the zinc depleted rats were due to zinc deficiency and not to differences in calorific intake.[39] Furthermore, Suwarnasarn et al. demonstrated that zinc-deficient rats compared with pair-fed rats had reduced epiphyseal plate height and retarded growth.[103] Similar results were reported by Dørup et al.[32]

Table 4. Final body weight (mean ± SD)

	Group 1	Group 2	Group 3
Zinc in diet (mg/kg)	2	47	60
Final body weight (g)	108 ± 13.3	218.3 ± 13.5[a]	276 ± 18.5[a,b]

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

Table 5. Animal body weight in the fracture study. (mean ± SD)

	Group 1	Group 2	Group 3	Group 4
Zinc in diet (mg/kg)	2	100	2	100
Healing time (days)	21	21	56	56
Body weight at:				
Start of experiment (g)	341 ± 11.2	341 ± 7.7	340 ± 11.2	341 ± 8
Operation (g)	375 ± 18.7	383 ± 19.4	367 ± 18.7	385 ± 12[a]
Sacrifice (g)	402 ± 22.4	412 ± 31.0	448 ± 37.40	466 ± 28
Body weight change during fracture healing (g)	27 ± 7.5	29 ± 19.4	81 ± 22.4	80 ± 16

Key: [a]Significantly ($p < 0.01$) different from group 3.

Table 6. The final femur length (mean ± SD)

	Group 1	Group 2	Group 3
Zinc in diet (mg/kg)	2	47	60
Femur length (mm)	27.22 ± 1.02	29.58 ± 0.83[a]	32.96 ± 0.83[a,b]

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

Todd et al. also found that the reduced growth and abnormal fur coat in the zinc-depleted animals were due to zinc-depletion and not due to reduced food intake.[108] Therefore, it is likely that the skeletal effects in the present study were caused by zinc depletion and not by the reduced food intake. The reason there were no significant differences in the growth of the rats in the fracture study could be that these rats responded by reduced growth more to the trauma (fracture), than to zinc-depletion. Another explanation could be that the animals were older at the initiation of paper III, than in papers I and II. Andreen and Larsson thus reported that old rats more readily go into negative zinc balance, than young rats, and that rats in negative zinc balance after fracture trauma are dependent on skeletal zinc stores to maintain homeostasis.[8] Other studies have shown that the zinc accumulation in intact bone in older rats is only approximately 10% of that seen in six weeks old rats.[8, 117] Mature rats do not absorb less zinc, than young rats, but secrete more endogenous zinc, initially by increased faecal excretion and later by marked increase in urinary zinc excretion.[8, 25, 68, 117]

3.3. External callus volume and dimensions

External callus diameters and external callus volume at 21 and 56 days of healing are given in Table 7. After 21 days of healing, no differences in external callus volume, anterior-posterior diameter, or medial-lateral diameter were seen between zinc deficient and zinc sufficient animals. In the zinc sufficient groups, the external callus volume and diameters in both anterior-posterior and medial-lateral dimensions were reduced from 21 days to 56 days of healing (31%, $p < 0.001$; 15%, $p < 0.001$; 19%, $p < 0.001$, respectively). In the zinc deficient groups, the external callus volume and diameters in both anterior-posterior and medial-lateral dimensions were also reduced from 21 days to 56 days of healing (47%, $p < 0.002$; 25%, $p < 0.001$; 22%, $p < 0.001$, respectively). Therefore, after 56 days of healing, no differences in external callus volume, anterior-posterior diameter, or me-

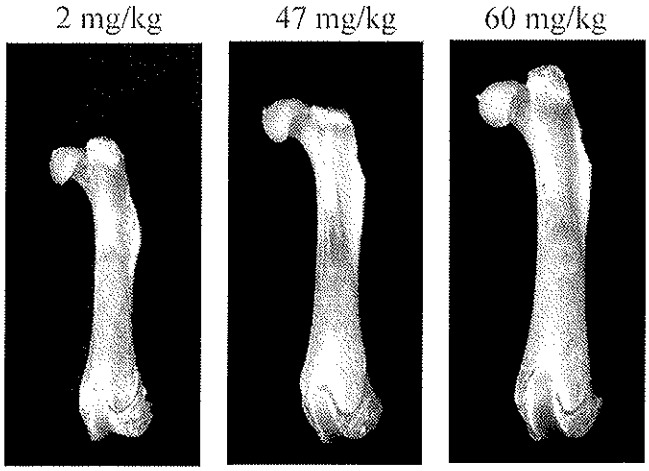

Figure 6. Examples of the femora from the three different groups.

Table 7. Dimensions and volume of fractured and contralateral intact tibia as well as body weights after 21 and 56 days of healing (mean ± SD).

	Group 1	Group 2	Group 3	Group 4
Zinc in diet (mg/kg)	2	100	2	100
Healing time (days)	21	21	56	56
Fractured tibia:				
Anterior-posterior (mm)	7.3 ± 1.1	6.8 ± 0.8	5.5 ± 0.7	5.8 ± 0.8
Medial-lateral (mm)	5.6 ± 0.4	5.6 ± 0.4	4.4 ± 0.7	4.5 ± 0.4
Volume (mm^3)	252 ± 112.2	211 ± 50.3	133 ± 41.2	146 ± 48
Tibia volume (mm^3)	766 ± 127.2	730 ± 69.7	647 ± 71.1	676 ± 56
Contralateral intact tibia				
Anterior-posterior (mm)	2.9 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	3.0 ± 0.08
Medial-lateral (mm)	2.6 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.16
Tibia volume (mm^3)	514 ± 41.2	518 ± 34.9	514 ± 44.9	530 ± 28

dial-lateral diameter were seen between zinc deficient and zinc sufficient animals.

From 21 to 56 days of healing the remodeling of callus tissue resulted in nearly identical external callus volumes in the two groups at 56 weeks of healing. Therefore, we conclude that alimentary zinc-depletion do not influence callus reduction. These findings are similar to those reported by Milachowski et al.[71] This could be explained by the fact that both the study by Milachowski et al. and the present study investigated young fractured rats, which have much higher zinc balances than old rats. This is in accordance with Andreen and Larsson reporting that old rats more readily go into negative zinc balance, than young rats, and that rats in negative zinc balance after fracture trauma are dependent on skeletal zinc stores to maintain homeostasis.[8] Other studies have shown that the zinc accumulation in intact bone in older rats is only approximately 10% of that seen in six weeks old rats.[8, 117]

3.4. Biomechanical tests

3.4.1. Three-point bending test of the femoral diaphysis (Papers I and III)

The results of the three-point bending test of the femoral mid-diaphysis of the growing rats (Paper I) are shown in Figure 7. Zinc supplementation of the growing rats influenced maximal load and axial moment of inertia in a dose-response pattern. The maximum load values of the femoral diaphysis in the zinc-supplemented groups were significantly ($p < 0.0001$) higher than the maximum load values of diaphyses from the zinc-deficient group. The maximum load values of 60 mg/kg zinc were also significantly ($p < 0.0005$) higher than the maximum load values of 47 mg/kg zinc.

The axial moment of inertia showed the same dose-response pattern as the maximum load values. The maximum stress values, however, showed no differences between the groups, indicating no differences in the quality of the bone tissue between the three groups. However, the Young's modulus values were significantly higher in the zinc-deficient group ($p < 0.005$) than the values of 60 mg/kg zinc. But no differences were seen between the two zinc-supplemented groups, indicating no differences in the stiffness of the bone in the two zinc-supplemented groups.

In the fracture study, after 21 days of healing, no differences in fracture strength (ultimate load, ultimate stiffness, and deflection at ultimate load) were found between zinc deficient and zinc sufficient animals. From 21 to 56 days of healing, a substantial enhancement of fracture strength was observed in both zinc sufficient animals (ultimate load: 650%, $p <$

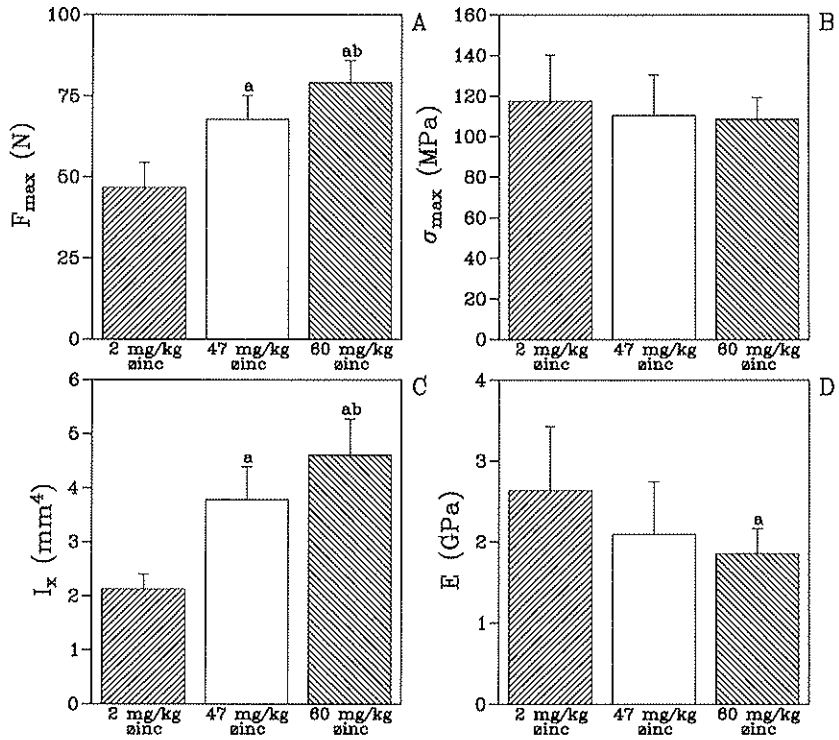

Figure 7. Femoral diaphysis. (A) Maximum load values; (B) axial moment of inertia; (C) maximum stress values; and (D) Young's modulus.[a]significantly different from the 2 mg/kg zinc group; [b]significantly different from the 47 mg/kg zinc group (mean ± SD).

0.001; ultimate stiffness: 930%, $p < 0.001$) and zinc deficient animals (ultimate load: 360%, $p < 0.001$; ultimate stiffness: 850%, $p < 0.001$). At 56 days of healing, ultimate load was significantly lower in the zinc deficient rats (20%, $p = 0.03$) than in the sufficient rats, whereas ultimate stiffness did not differ significantly (14%, $p = 0.14$) between the two groups. The results are given in Figure 8.

From 21 to 56 days of healing a substantial enhancement of callus mechanical quality was observed in both groups of rats. However, a limited but significant decrease in fracture strength was observed in the zinc-deficient group of rats. To the best of our knowledge no previously published studies have investigated the effects of alimentary zinc on bone strength of healing fractures and only one in vivo

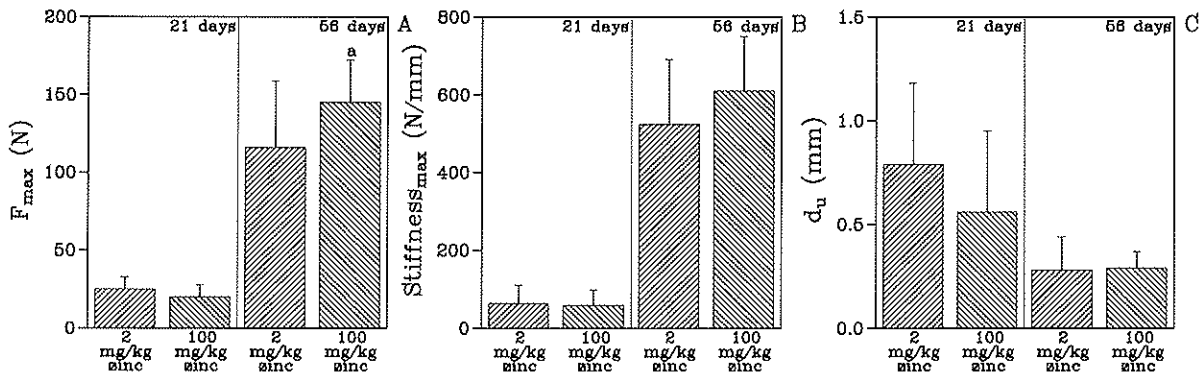

Figure 8. Mechanical properties of fractured tibia after 21 or 56 days of healing (mean ± SD). [a]significantly different from the zinc-deficient group at 56 days. (A) maximum load values; (B) maximum stiffness; (C) deflexion at fracture.

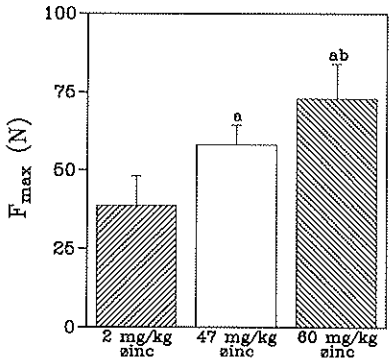

Figure 9. Femoral neck. Maximum load values. [a]significantly different from the 2 mg/kg zinc group; [b]significantly different from the 47 mg/kg zinc group (mean ± SD).

study has shown stimulatory effect of zinc administration on fracture healing in rats: Igarashi et al. thus found that administration of zinc acexamate (10 mg Zn/100 mg) for 28 days caused a significant increase in calcium content, alkaline and acid phosphatases activities, and protein and DNA contents in the tissues of rats after fracture healing.[49] Apart from that study by Igarashi et al. only in vitro studies have reported stimulatory effect of zinc on fracture healing in young rats.[48]

3.4.2. Femoral neck (Paper I)

All femora fractured with a transcervical fracture close to the diaphysis. The maximal load values of the femoral neck were significantly higher in both zinc-supplemented groups compared with the zinc-depleted group. The maximum load values of 60 mg/kg zinc animals were also significantly higher than the maximum values of 47 mg/kg zinc animals ($p < 0.0005$). Results are shown in Figure 9.

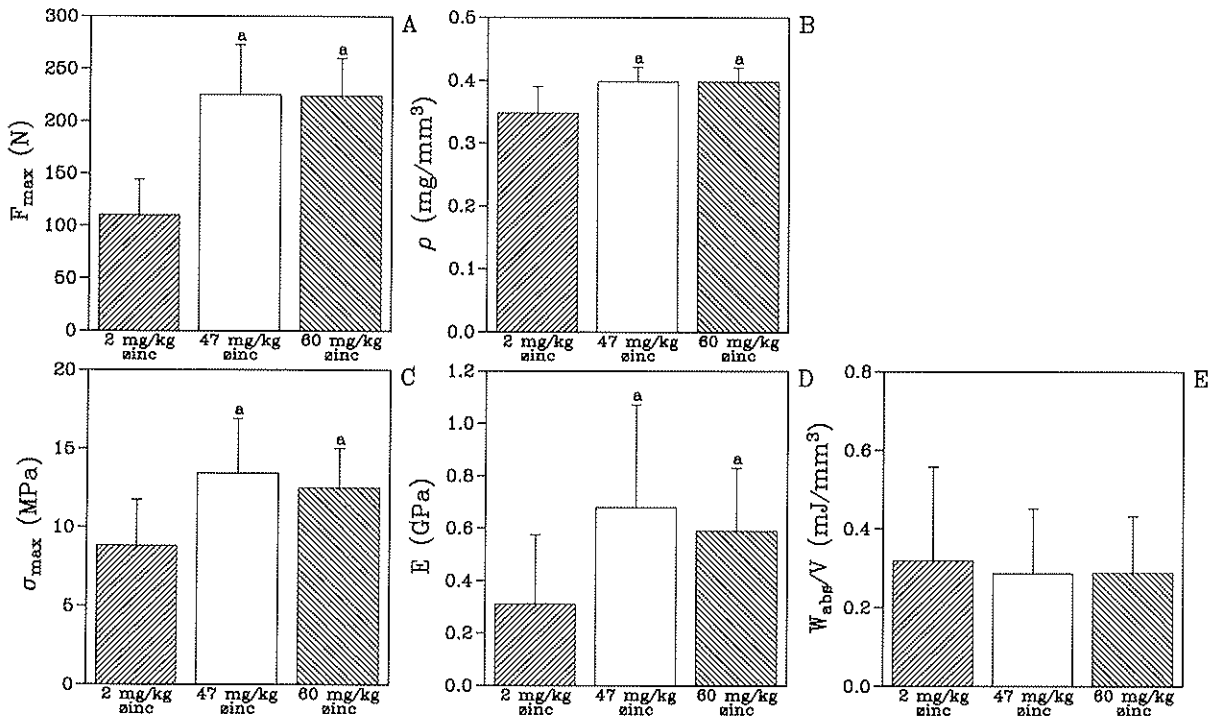

Figure 10. Distal femoral metaphysis. (A) Maximum load values; (B) ash density; (C) maximum stress values; (D) Young's modulus; and (E) Energy absorption normalized with bone volume. [a]significantly different from the 2 mg/kg zinc group; [b]significantly different from the 47 mg/kg zinc group (mean ± SD).

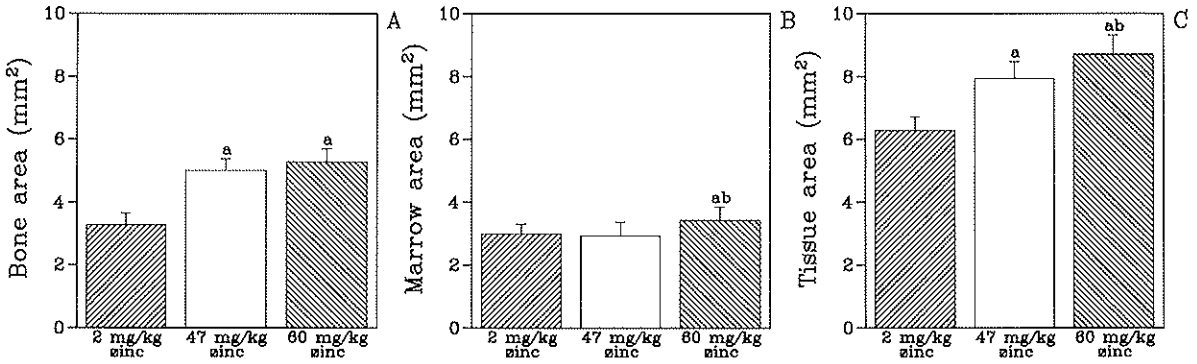

Figure 11. Femoral diaphysis area. (A) Bone area; (B) marrow area; and (C) tissue area. [a]significantly different from the 2 mg/kg zinc group; [b]significantly different from the 47 mg/kg zinc group (mean ± SD).

3.4.3. Distal femoral metaphysis (Paper I)

The maximum load values of the femoral metaphysis were significantly higher in the zinc-supplemented groups compared with the zinc-depleted group, but no dose-dependent response was seen at this site. The ash densities, Young's modulus and the maximum stress values exhibited a similar pattern of response to the different zinc content as the maximum load values at the distal femoral metaphysis (Figure 10).

The findings in paper I showed, that zinc influenced bone strength in growing rats in a dose-response pattern at all investigated sites except at the distal femoral metaphysis. These findings suggest that the bone abnormalities in zinc-deficient rats are caused not only by changes in the growth plate cartilage as mentioned in several studies [46, 56, 61, 63] but also by changes in the bone size (Figure 11). The positive effects of zinc on bone mass in this study did not, however, seem to have a negative influence on bone quality, because the maximum stress (the maximum compressive load normalized for bone size) was unchanged in the femoral diaphysis and increased in the distal metaphysis. The unchanged maximum stress values in the diaphysis and the energy-absorption (normalized for bone size) are a direct indication that tissue quality is unchanged in the three groups. This is contrast to a previously study by Søgaard et al. with fluoride, in which an increase in bone mass did not translate into an increase in bone strength.[105] Zinc, therefore, seems to have a positive effect on the skeleton that is totally different from that of fluoride, but nearly the same as that shown for PTH, GH, and vitamin D analogues with the formation of new bone of normal biomechanical competence.[4, 5, 6, 38, 75, 76, 78, 79, 107]

To our knowledge, this is the first time the effects of dietary zinc on bone strength in growing rats have been investigated in a dose-response manner. Suwarnasarn et al. reported that the force to displace the epiphysis of zinc-deficient rats was always less than that required for pair-fed rats.[103]

3.5. Static histomorphometry

The results revealed that zinc exerted its main effects on the periosteal envelopes, mainly causing increased bone area and tissue area, and axial moment of inertia. The effect of zinc thereby mimicked the previously described effect of growth hormone or insulin-like growth factor 1 (IGF-I).[6, 79]

A few other pharmaceutical agents are known to have anabolic effects on bone metabolisms, among them parathyroid hormone (PTH).[6, 75, 76, 77, 79, 107] But PTH is, unlike zinc, known to act on both envelopes, but mainly the endocortical envelope in rats.[107] The results are shown in Figure 11 and 12.

The net amount of bone is determined by the difference between the amount of bone removed from the endocortical, trabecular and

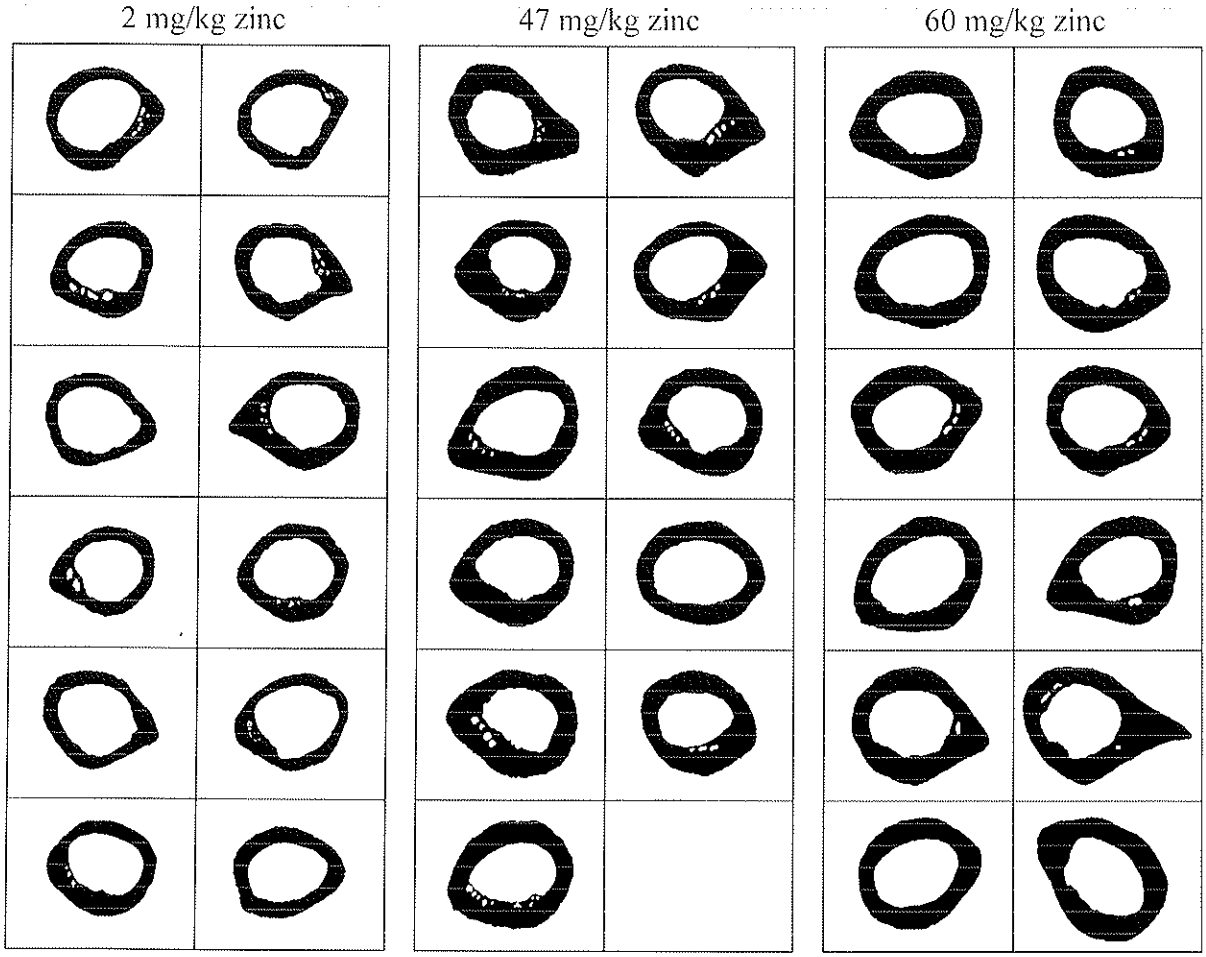

Figure 12. Mid-diaphyseal cross sections of the individual animals obtained as close as possible to the fracture point.

intracortical components of its endosteal (inner) envelopes (remodeling) and the amount formed beneath its periosteal (outer) envelope (modeling).[79]

Eberle et al. reported the skeletal effects of alimentary zinc deficiency in growing rats using quantative bone histomorphometry. They found that in the distal femoral metaphysis zinc deficiency led to a 45 % reduction in cancellous bone mass, osteopenia and to a deterioration of trabecullar bone architecture, with fewer and thinner trabeculae. Thus, suggesting that the osteopenia observed in zinc deficient rats can be characterized as a low turnover osteopenia, i.e. as an osteopenia that is accompanied by a reduction in both osteoblastic bone formation and osteoclastic bone resorption.[33]

In young individuals as in our study the net amount of bone loss is very slow because of the very low remodeling rate. We believe from our results in growing rats, that zinc has an anabolic effect on bone metabolism with its main effect on the periosteal envelopes. However, future histomorphometric studies using in vivo fluorochrome labelling of the mineralization front are needed to elucidate further the role of zinc deficiency on bone growth and mineralization.

Figure 12 shows the mid-diaphyseal cross sections obtained as close as possibly to the fracture site. The differences in especially tissue area revealed in Figure 11 can easily be appreciated from Figure 12.

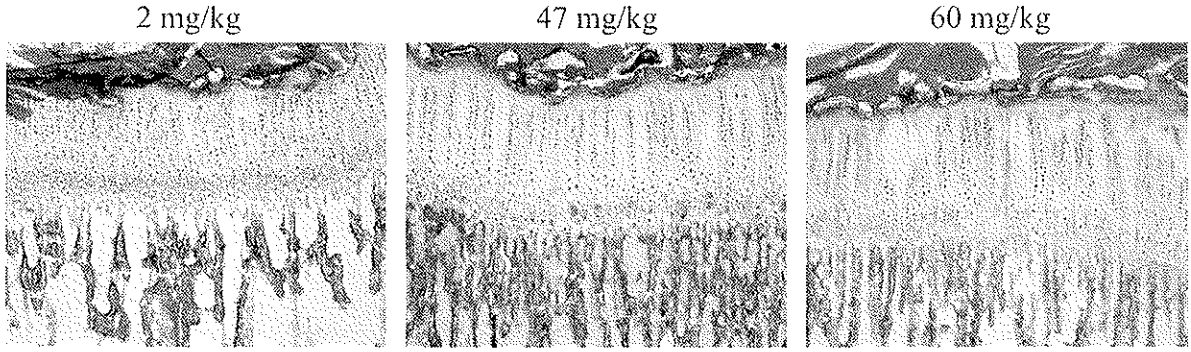

Figure 13. Examples of the growth plate from the three different groups.

3.6. Histology

3.6.1. The growth plate height (Paper II)

The study showed significant differences in the height of the growth plate of the animals fed a diet of 2 mg/kg zinc, 47 mg/kg zinc, and 60 mg/kg zinc. Figure 13 shows an example of the growth plate from each of the groups. The growth plate heights followed a dose-dependent pattern with respect to the zinc content of the diet fed to the rats (Figure 13 and 14). The majority of this increase in growth plate height was accounted for by an increase in the height of the hypertrophic zone and the irregularly-shaped lacunae, with lack of the straight columnar arrangement in the hypertrophic layer in the groups supplemented with zinc compared with the zinc-depleted group.

These results are similar to those reported by Wang et al. showing, that zinc deficiency inhibits proliferation and differentiation of growth plate chondrocytes.[114] Rodriguez and Rosselot demonstrated that zinc supplementation increased the proliferation rates of proliferating epiphyseal chondrocytes.[98] These results 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 growth.[47]

3.6.2. ZnS^{AMG} detection of zinc ions (Papers II and III)

3.6.2.1. Bone tissue

The ZnS^{AMG} grains were concentrated intracellularly in the osteoblasts and osteocytes and extracellularly in the osteoid in all 3 groups. The AMG grains were present in a relatively large number in the mineralizing ostoid 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.

Not all osteocytes contained AMG grains,

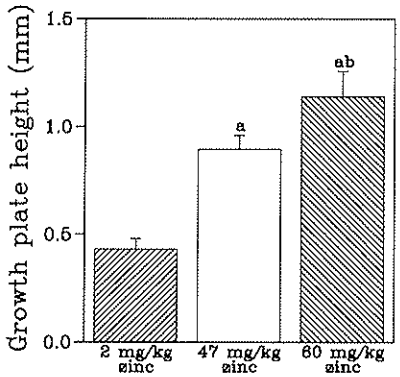

Figure 14. The growth plate height (mean ± SD). [a]Significantly different from the 2 mg/kg zinc group. [b]Significantly different from the 47 mg/kg zinc group.

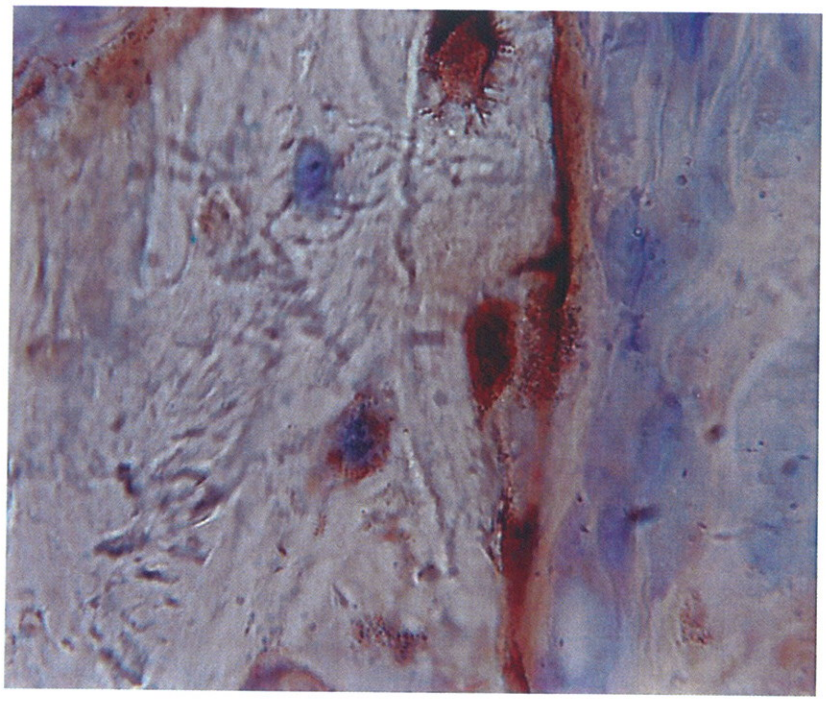

Figure 15. Osteocytes with osteocytic processes loaded with AMG grains.

but in some areas, especially near bone surfaces (endocortically and periosteally), the osteocytes demonstrated AMG grains along their osteocytic processes (Figure 15). ZnS^{AMG} grains were also found extracellularly on the longitudinal partitions running between the hypertrophic chondrocytes, i.e. in places where calcification of matrix is known to commence. In contrast, the mineralized bone was void of ZnS AMG grains. In addition, it was not possible to demonstrate any differences in the concentration or pattern of the AMG grains in the 3 groups.

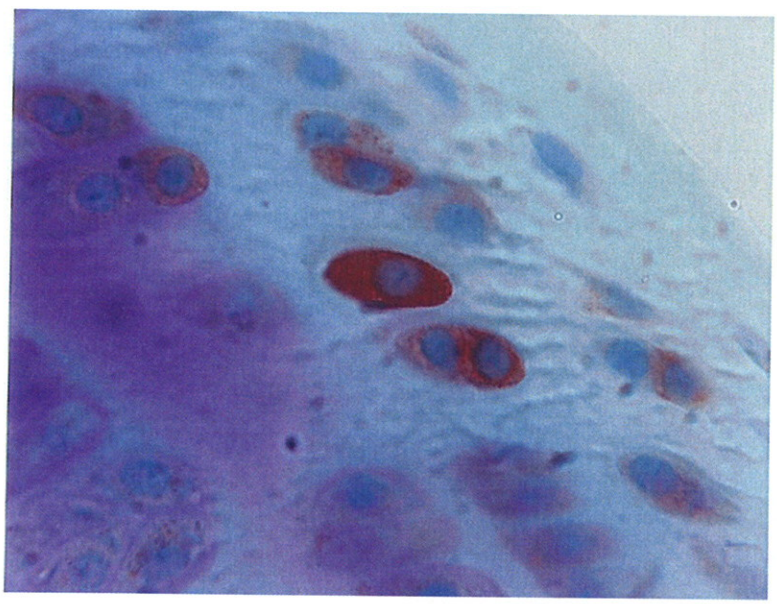

Figure 16. AMG grains in the cytoplasma of the chondrocytes of the articular cartilage.

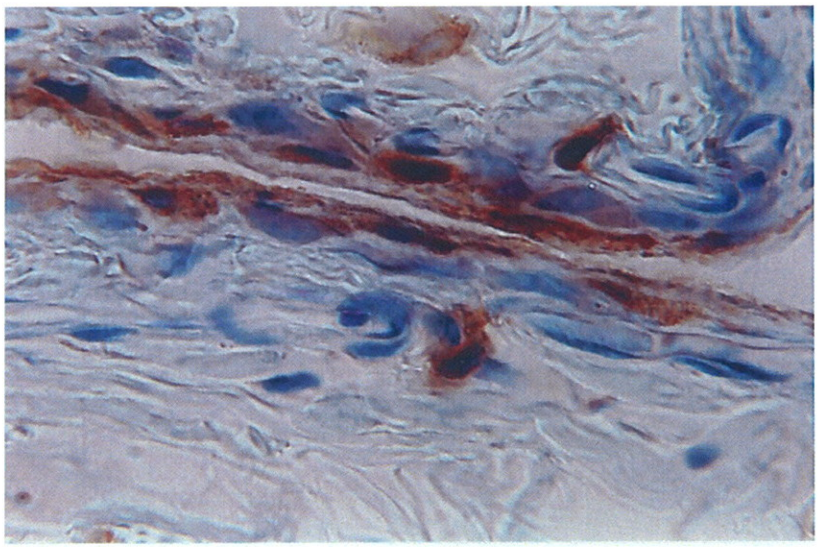

Figure 17. AMG grains in the cytoplasma of the ellipsoidal cells of the synovial membrane.

3.6.2.2. 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 proliferation zone had the highest content of AMG grains (Figure 16). Several authors have isolated chondrocytes from the growth plate and suggested zinc as a regulator of calcification,[46, 56] but to the best of our knowledge nobody has previously located Zn ions in the chondrocytes of the articular cartilage. The present study has demonstrated the presence of ZnS AMG grains both in the superficial layer, and also in the deeper, partially-calcified layer of the articular cartilage. Therefore, we suggest that the zinc ions may be important in the regulation of the calcification of cartilage.

3.6.2.3. Synovial membrane

The one to four cells-thick layer of ellipsoidal cells of the synovial membrane contained

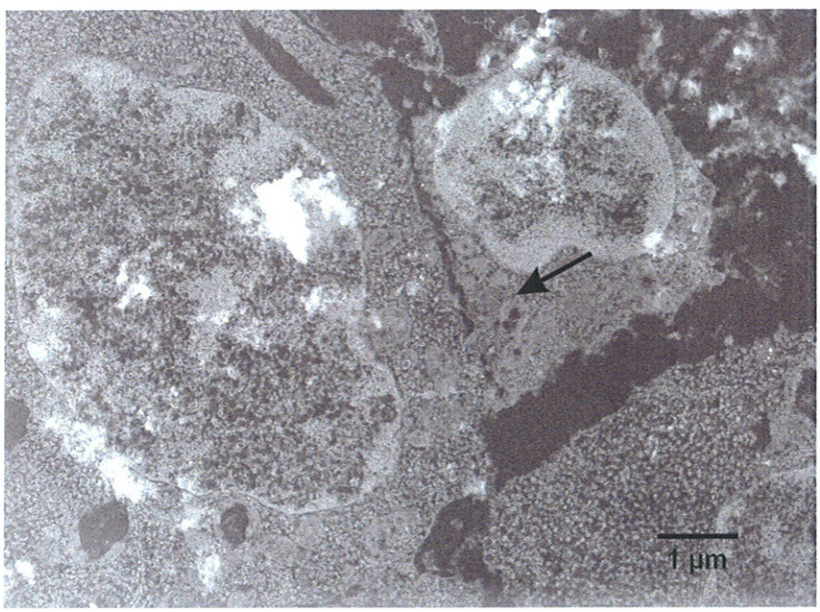

Figure 18. Osteoblast in an area of ostoid with diffuse AMG staining in small cytoplasmic vacuoles.

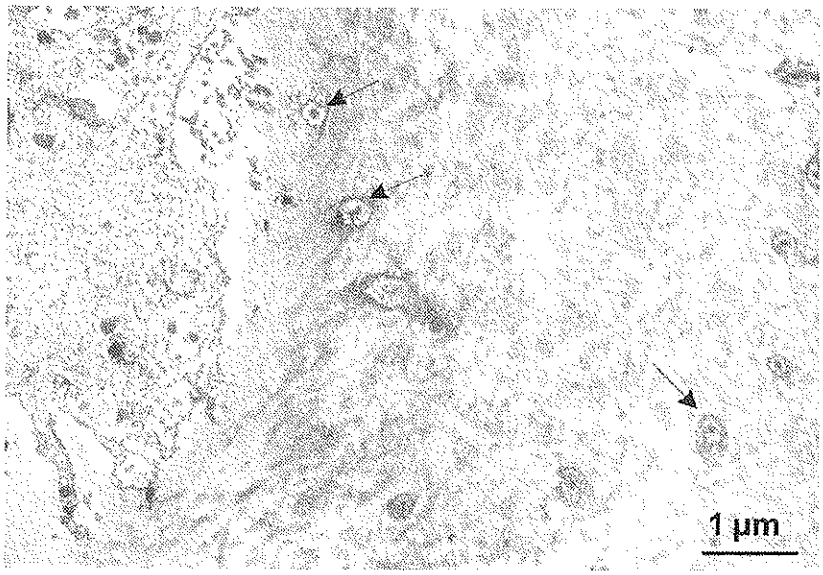

Figure 19. Border between mineralized and unmineralized bone matrix. The AMG grains are associated with vesicular structures in the mineralizing bone, probably matrix vesicles.

AMG grains in their cytoplasma and especially in the inner layer of the membrane (Figure 17). The localization of zinc ions in the upper epithelial cell layer of the synovial membrane cannot be explained, but it might indicate that synovia contains free zinc ions, secreted from these cells which are important to the nourishment of the joint cartilage. The major nutrition of the joint cartilage is by environment diffusion, especially from the synovial liquid.

3.6.2.4. Ultrastructural level

A few of the bone tissue blocks from the distal part of the femoral metaphysis were embedded in Epon and examined in an electron microscope. AMG grains are seen in small cytoplasmic vacuoles in the osteoblasts and in the osteoblastic osteocytes located in the deeper layer of the unmineralized matrix. The AMG grains located here are related to matrix vesicles (Figures 18 and 19).

In growing rats, calcified cartilage serves as a template for bone formation (i.e., endochondral ossification). Matrix vesicles have been described to be involved in the induction of calcification on growth plate cartilage (hypertrophic zone). Anderson described the matrix vesicles as the initial site of calcification and that their biogenesis in the growth plate is linked to the chondrocyte cell cycle.[3] This mechanism is described as a biphasic event: Phase 1 is initiated by cells generating calcifiable matrix vesicles and releasing them into sites of intended calcification. Phase 2 begins with breakdown of matrix vesicle membranes, exposing hydroxyapatite to the extracellular fluid, after which mineral crystal proliferation is regulated by extracellular conditions. This hypothesis has been supported by others.[23, 45, 46, 47, 56, 99]

Our results confirmed the present of zinc ions in non-mineralized osteoid and osteoblasts at the site of bone formation. At ultrastructural levels (electron microscopy) we found AMG-stained zinc ions that we believe to be matrix vesicles in the unmineralized bone matrix. Matrix vesicles because of the size of the particles and also the visible sap, a needle like dense crystal of hydroxyapatite mineral.[3] These findings are consistent with the findings of Dancher et al.[28]

3.6.2.5. Fractures

After 21 days the specimens from the zinc deficient group seemed to produce a more

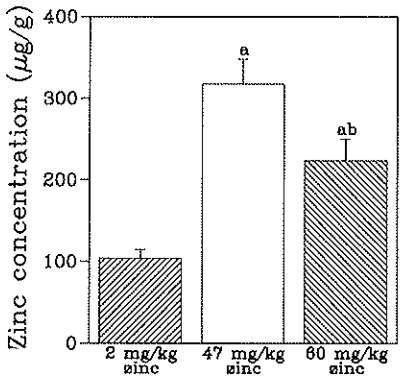

Figure 20. Zinc concentration in femoral bone. [a]significantly different from the 2 mg/kg zinc group; [b]significantly from the 47 mg/kg zinc group.

proliferative procallus than the zinc supplemented group. These differences could not be found after 56 days of healing. In spite of this, there were no histological differences between the corresponding procallus and callus of the two groups.

The AMG-traced zinc was concentrated in the osteoid in both groups and it was not possible to demonstrate any differences in the concentration or pattern of the AMG grains in the two groups. Furthermore, the procallus and callus were void of ZnS^{AMG} grains.

3.6.3. Controls (Papers II and III)

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. The same was found for control sections from animals which had not been perfused with sodium sulphide.

3.7. Concentration of Zinc in intact bone (Papers I and II)

The left femora from the three groups of rats that received a semisynthetic diet with differing amounts of zinc added were ashed, dissolved in HCL and the concentrations determined by a spectrophotometer. The results are presented in Figure 20.

The zinc concentration in the femora of the 2 mg/kg zinc group showed significantly lower amounts than for the two other groups. However, the values in the 47 mg/kg zinc group showed significantly higher amounts of zinc concentration than the 60 mg/kg zinc group. An explanation could be that this is the concentration of the whole femora and that this whole bone in the 60 mg/kg zinc group is much larger than in the 47 mg/kg zinc group.

Several authors have described that the body makes homeostatic adjustments in digestion and retention of zinc for a balanced supply to the tissues and organs according to their specific needs.[18, 25, 68, 115, 116] Especially the zinc concentration in bone is markedly influenced by the zinc level in the diet. In most studies, bone was analyzed as a whole bone, as we did. Their results were comparable to ours; Swenerton and Hurley[104] found a zinc concentration in the femur of 126 µg/g in the zinc-sufficient group and 14 µg/g in the zinc-deficient group, Prasad et al.[92] found zinc-sufficient 168 µg/g, zinc-deficient 69 µg/g, and Williams and Mills[116] recorded zinc-sufficient 117 µg/g, zinc-deficient 95 µg/g. Bergman was the first to analyze various portions of the bone specimen and showed a decrease in the zinc content of the tibia epiphysis in zinc-depleted rats, compared with zinc-supplemented rats, but found no differences in the tibial diaphysis.[18] This suggests that the epiphyseal growth plate is the most sensitive part of the bone to zinc deficiency.

There are a number of studies of the zinc concentration in human bone related to age and bone strength.[10, 35, 72] Alhava and al. thus showed, that zinc content of the bone influenced bone strength of cancellous bone from the iliac crest in both men and women.[2]

Two mechanisms could be responsible for the inhibition of zinc deficiency on the growth plate activity. One possibility is that the zinc ions are not locally available for the chondrocyte proliferation and maturation at the growth plate. It has been shown, that zinc deficiency inhibits the proliferation of chondrocytes[114] and that zinc supplementation stimulates the proliferation of epiphyseal growth plate chondrocytes.[98] Our results confirm this mechanism of zinc by the presence of a high numbers of zinc ions in the non-mineralized osteoid and osteoblasts. These findings are in accordance with the results of Calhoun et al. who found that zinc is required at the site of bone formation as a requisite for complete calcification.[22]

But this direct effect of zinc on the growth plate cannot explain our results of zinc concentration in the femora of the three different groups. Therefore zinc deficiency seem to have another alternative mechanism, which is that zinc indirectly affects cellular activities in the growth plate through changing levels of hormones and growth factors. Studies have shown that zinc deficiency reduces GH and IGF-I levels.[73] These findings suggest that zinc affects longitudinal bone growth through the GH-IGF-I system. However, if the serum concentration of IGF-I was restored by IGF-I infusion to the zinc-depleted rats, this did not reverse growth retardation. These results suggest that growth retardation related to zinc deficiency cannot be explained only by the actions of GH and IGF-I.[20, 31, 32, 84] It has therefore been suggested that zinc in some way is essential for IGF-I induction of cell proliferation and transforming growth factor β (TGF-β) in osteoblastic cells in vitro.[66, 73]

Chapter 4: Conclusions

1. Alimentary zinc supplementation in growing rats has a potent anabolic effect. Both the body weights and the length of the femora increased dose-dependently.
2. Zinc induced a significant increase in bone strength in growing rats at all investigated sites.
3. Zinc influenced bone strength in growing rats in a dose-dependent manner except at the distal femoral metaphysis, where there was no significant difference between the rats fed with the diets with the two highest zinc content.
4. Static histomorphometry showed that zinc exerted its main effect in growing rats on the periosteal envelope.
5. Alimentary zinc supply to growing rats resulted in an increase in the height of the total growth plate in a dose-dependent manner.
6. Based on the modified autometallographic ZnS^{AMG} technique, zinc ions are located in osteoid bone, joint cartilage and synovial membrane.
7. At ultrastructural levels (electron microscopy) AMG-stained zinc ions were traced to the initial site of calcification i.e. what we believe to be matrix vesicles in the unmineralized bone matrix.
8. Zinc concentration in bone in growing rats was significantly decreased in zinc depleted rats.
9. The growth in growing rats suffering a fracture was not influenced by the zinc content of the daily diets.
10. The time of fracture healing in growing rats was not influenced by the additional administration of zinc to the daily diets and no histological differences were found.
11. Biomechanical testing revealed a significant zinc-induced effect on the maximum load values of the fracture site after 56 days of healing in growing rats.

Chapter 5: Suggestions for Future Research

The present study analyzed the effects of zinc ions on bone growth and fracture-healing in weaned rats. However, these relations might not be the same in older individuals. It would therefore be interesting to evaluate whether supplemental zinc could be beneficial for bone healing and in preventing the development of osteopenia and osteoporosis in older rats. An investigation with a longer preoperative period of zinc-depletion/supplementation, perhaps with a higher administrated dose of zinc, would be interesting to perform in relation to bone fractures. Judging from the results obtained in the fracture study, a more pronounced effect would probably be seen. It seems reasonable to conduct investigations of the effects of zinc on bone modeling and remodeling in human. We had planned to take out biopsies from the growth plate from children going through an osteotomy/epiphysiodesis, and the study was approved by the Medical Ethical Committees of Aarhus. However, during the last years only a few children have been operated this way because of introduction of new techniques e.g. the Ilizarow technique, so it would be hard/impossible to obtain enough patients for such an investigation. Furthermore, we are at present dealing with methodological improvements of AMG in order to examine these biopsies.

A similar study in older humans suffering an osteoporotic fracture also seems appropriate.

Chapter 6: Summary

This Ph.D.-thesis is based on three original papers investigating the effects of zinc on bone modeling and remodeling.

Aims

I. Investigation of the bone quality after alimentary zinc depletion and supplementation in an animal model of intact, growing rats. A biomechanical study. Paper I.
II. A histological description of bone changes and the amount and localization of zinc ions following alimentary zinc depletion and supplementation in an animal model of intact growing rats. A histological study. Paper II.
III. The effects of alimentary zinc depletion and supplementation on fracture healing in an animal model with a standardized closed fracture. A biomechanical and histological study. Paper III.

Methods

Forty-five, 4-weeks-old, male Wistar rats were used for the investigations in paper I and II, and eighty, 12-weeks-old male rats were used in the fracture-study (paper III). 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 a semisynthetic diet with different amounts of zinc added and distilled water. All animals were sacrificed and both hindlimbs were investigated by use of histology and biomechanical testing.

Results and conclusions

Paper I and II

- Alimentary zinc supplementation in growing rats has a potent anabolic effect. Both the body weights and the length of the femora increased dose-dependently.
- Zinc induced a significant increase in bone strength in growing rats at all investigated sites.
- Zinc influenced bone strength in growing rats in a dose-dependent manner except at the distal femoral metaphysis, where there was no significant difference between the two groups of rats fed with zinc-supplemented diet.
- Static histomorphometry showed that zinc exerted its main effect on growing rats on the periosteal envelope.
- Alimentary zinc supply to growing rats resulted in an increase in the height of the total growth plate in a dose-dependent manner.
- Based on the modified autometallographic Timm sulphide silver method (AMG), zinc ions are demonstrated as present in osteoid bone, joint cartilage and synovial membrane. In other words at the site of bone formation as a requisite for complete calcification.
- At ultrastructural levels (electron microscopy) AMG-stained zinc ions were demonstrated at the initial site of calcification in, what we believe to be, matrix vesicles in the unmineralized bone matrix.
- Zinc concentration in bone in growing rats was significantly decreased in zinc depleted rats.

Paper III

- The growth in growing rats suffering a fracture was not influenced by the

zinc content of the daily diets.

- The time of fracture healing in growing rats was not influenced by the additional administration of zinc to the daily diets and no histological differences were found.
- Biomechanical testing revealed a significant zinc-induced effect on the maximum load values of the fracture site after 56 days of healing in growing rats.

Chapter 7: Dansk Resumé

Ph.D. afhandlingen er baseret på tre eksperimentelle studier udført ved Anatomisk Institut; Århus Universitet og Ortopædkirurgisk afdeling, Århus Universitetshospital.

Formål

I. At bestemme knoglekvaliteten efter forskelligt zink-indtag i en rottemodel af unge, voksende rotter. Et biomekanisk studie. Paper I.
II. En histologisk beskrivelse af knogleforandringerne, zink-indholdet og zinklokalisationen i en rottemodel behandlet med forskellige doser af zink via kosten. Et histologisk studie. Paper II.
III. At bestemme effekten af forskelligt kostindtag af zink på frakturheling i en rottemodel med en standardiseret knoglefraktur. Et biomekanisk og histologisk studie.

Metode

Fire uger gamle Wistar, han-rotter (N=45) blev brugt til undersøgelserne i studie I og II. Tolv uger gamle Wistar han-rotter (N=80) indgik i frakturstudiet (paper III). Dyrene blev anbragt parvis i metalfri-bure i rum med en kontrolleret temperatur (21 ± 2°C) og en 12:12 timers dag/nat cyklus. Dyrene havde fri adgang til destilleret vand og et semisyntetisk fremstillet foder med forskelligt indhold af zink. Alle dyrene blev aflivet og underekstremitets knoglerne indgik i histologiske og biomekaniske undersøgelser.

Resultater og konklusioner

Paper I and II

- Zink-tilskud i kosten til voksende rotter har en potent anabolisk effekt. Både kropsvægten og længden af femora steg dosis-afhængigt med zinktilskuddet.
- Zinktilskud medførte en signifikant øgning i knoglestyrken på alle undersøgte anatomiske steder hos voksende rotter.
- Knoglestyrken hos voksende rotter steg dosisafhængigt undtagen svt den distale femorale metafyse, hvor der ikke blev fundet signifikant forskel imellem de to grupper, som fik tilsat zink i kosten.
- Statisk histomorfometri viste at zinks primære effekt hos voksende rotter var svarende til den periosteale envelope.
- Zinktilskud til voksende rotter medførte en dosisafhængig højdeøgning af epifyseskiven.
- Baseret på Timm's modificeret autometallografiske sulfid sølv metode (AMG), demonstrerede vi zink i det osteoide væv, ledbrusken og synovialmembranen.
- Elektronmikroskopiske undersøgelser af knoglepræparater viste AMG-sølvfarvet zinkioner i matrixvesikler lokaliseret i den umineraliseret knoglematrix.
- Zink koncentrationen i knoglevævet hos voksende rotter var signifikant lavest i de dyr der fik mindst zink i kosten.

Paper III

- Væksten hos voksende rotter udsat for en standardiseret fraktur var ikke afhængig af zink indholdet i kosten.
- Frakturhelingsperioden hos voksende rotter var ikke afhængig af zink indholdet i kosten. Der blev ikke fundet histologiske forskelle omkring frakturerne.

- Biomekaniske tests viste signifikant forskelle på knogle styrken efter 56 dages frakturheling hos voksende rotter.

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Appendix

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Bone Vol. 29, No. 6
December 2001:565–570

The Positive Effects of Zinc on Skeletal Strength in Growing Rats

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The aim of the present study was to assess the skeletal effects of alimentary zinc depletion and supplementation in an animal model of intact, growing rats. The study was planned as a dose-response study. Thirty-six male Wistar rats, 4 weeks old, were divided into three groups of 12 rats each. The rats had free access to a semisynthetic diet with different amounts of zinc added. Group 1 was given a zinc-free diet containing 2 mg zinc/kg, group 2 was given a normal-zinc diet containing 47 mg zinc/kg; and group 3 was given a zinc-supplemented diet containing 60 mg zinc/kg. All animals were killed 4 weeks after initiation of the experiment and the right femora were removed. The biomechanical effects were measured at the following skeletal sites: femoral diaphysis; femoral neck; and distal femoral metaphysis. In addition, static histomorphometry was performed at the middiaphyseal region. Biomechanical testing revealed a significant zinc-induced increase in bone strength at all sites investigated. It also showed that zinc influenced bone strength in a dose-dependent manner except at the distal metaphysis, where there was no significant difference between the group fed normal-zinc diet and the group fed a hyper-zinc diet. Zinc also improved the rates of growth in the rats. The body weights and length of femora increased dose-dependently. Static histomorphometry showed that zinc exerted its main effect on the periosteal envelope, thereby increasing bone area, tissue area, and axial moment of inertia. We conclude that alimentary zinc supplementation in growing rats induces an increase of bone strength in both the femoral neck and the femoral diaphysis. These results further support the view that zinc has a positive effect on bone metabolism which mimicks that of growth hormone (GH) or insulin-like growth factor 1 (IGF-1). (Bone 29:565–570; 2001)

Key Words: Rat model; Zinc depletion; Osteopenia; Bone mass; Bone strength; Zinc supplementation.

Introduction

Zinc has been demonstrated to be essential for normal growth of the human skeleton and also for skeletal growth in many animals.[4,7,12,34,43,46] Zinc is a component of alkaline phosphatase (ALP) and several metalloproteases. ALP dysfunction has therefore been the traditional explanation for bone diseases associated with zinc deficiency. However, a number of in vitro effects of zinc ions on bone tissue cultures cannot be explained by the presence of zinc in ALP.[18,25,28] Previous studies have suggested that vitamins (vitamin A and vitamin D) play a role in the metabolism of bone under the regulation of zinc ions.[3,7] Other studies suggest that zinc affects normal cell proliferation in response to IGF-1 and thereby also skeletal growth.[27]

Clinically, zinc deficiency is known to be associated with retarded growth, dermal lesions, alopecia, and hypogonadism. Congenital skeletal disorders, spontaneous abortion, and fetus mortus are prominent effects of maternal zinc deficiency.[7,43] It has also been suggested that zinc plays an important role in the development of osteopenia and osteoporosis[2,10,17,37,38] and ostoporotic fractures.[13] At the tissue level, several studies have described the effects of zinc on bone histomorphometry and bone metabolism in various rat models.[12,20,21,39,44,46]

Recently, the existence of two pools of zinc in bone tissue from growing rats was suggested by histochemical detection of free/loosely bound zinc ions in osteoid and osteoblasts. The zinc ions were ultrastructurally traced in matrix vesicles located in the undecalcified bone and in osteoblasts.[9]

Because zinc seems to have effects on growth, bone turnover, and mineralization, the purpose of the present study was to characterize further the importance of zinc on bone quality in growing rats. To our knowledge, this is the first time the effects of dietary zinc depletion and supplementation on bone strength in growing rats have been investigated in a dose-response manner.

Materials and Methods

Animals and Diets

Thirty-six healthy male Wistar rats, 4 weeks old (Møllegaard's Breeding Center, Ltd., Ejby, Denmark), were randomly divided into three groups of 12 each. They were housed in pairs in metal-free cages in rooms with a controlled temperature (21 ± 2°C) and given free access to food and distilled water. The three groups received a semisynthetic diet (Altromin [special recommended diet for laboratory rodents], Brogården, Gentofte, Denmark) with differing amounts of zinc added: group 1 (n = 12) received a zinc-free diet containing 2.042 mg zinc/kg (hypo-zinc); group 2 (n = 12) received a diet containing 47 mg zinc/kg (control) (which is the normal level of dietary zinc in our standard rat food); and group 3 were fed a diet supplemented to 60 mg zinc/kg (hyper-zinc). The three diets were chosen to determine whether there was any dose-dependent influence of zinc on bone strength. All animals survived for 4 weeks, they

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8756-3282/01/$20.00
PII S8756-3282(01)00616-0

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were then killed by decapitation under anesthesia with Mebumal (50 mg/mL). At death, both hindlimbs were dissected from the body and all extraneous tissue was removed. The bones were then sealed in plastic wrap and immediately frozen at −20°C.

Biomechanical Testing

On the day of testing, the right femora were slowly thawed at room temperature. The specimens were placed in Ringer solution for 1 hr before the following biomechanical tests were conducted.

Three-point bending test of the femoral diaphysis. The length of the femora was measured with an electronic caliper, and the midpoint was marked with a waterproof marking pen. The femora were placed in a testing jig constructed for three point bending test. The distance between the supporting rods had a fixed length (L) = 15.7 mm. Load was applied at a constant deformation rate of 2 mm/min with a rod at the midpoint of the femur in a materials-testing machine (Alwetron TCT5; Lorentzen and Wettre, Stockholm, Sweden). Load-deformation curves were recorded and analyzed by personal computer (PC; ProLinea 4/33; Compaq, Houston, TX). The calculation of the biomechanical parameters was performed in accordance with Turner and Burr.[42] The following parameters were presented for the three point bending: maximal load, F_{max} (N); axial moment of inertia, I_x (mm^4); and maximum stress, σ_{max} (MPa).

Biomechanical testing of femoral neck. The proximal femora were mounted in a device for standardized fixation designed to produce a cervical, extracapsular fracture of the femoral neck. The fixation device holding the specimen, at an angle of 78°, was placed in the materials-testing machine and fastened with clamps. A vertical load conducted by a cylinder was applied to the top of the femoral head. The cylinder was moved at a constant rate of 2 mm/min until fracture of the femoral neck. The specimens were kept wet during the whole testing procedure. During compression, load-deformation values were obtained by a PC. Data presented include maximum load (F_{max}) N.

Biomechanical testing of the femoral metaphysis. From each femora an approximately 3.9-mm-thick section with planoparallel ends was sawed from the distal part of the metaphysis just above the anterior upper most part of the patellofemoral joint cartilage. The sections were sawed with a diamond precision-parallel saw (Exakt; Apparatebau, Otto Hermann, Nordstedt, Germany). The volume of the sections was measured by weighing the specimens before and during immersion in water on an electronic scale (Mettler AG 245; Mettler-Toledo, Nänikon-Greifensee, Switzerland) equipped to measure volumes. The distal femoral "cylinders" were then tested along the proximo-distal axis in the materials-testing machine at a constant deformation rate of 2 mm/min. During compression, load-deformation curves were recorded and later analyzed by PC. After compression, the specimens were ashed (105°C for 2 h and 580°C for 24 h). The ash weight was measured on the electronic scale, and the ash density was calculated as the ash weight divided by the volume of the cylinder. Data presented include maximum load (F_{max}; N), ash density; (ρ; mg/mm^3), and maximum stress (σ_{max}; MPa).

Static Histomorphometry

A 200-μm-thick section was sawed off the proximal part of the femoral diaphysis as close as possible to the fracture point from the three point bending. Before sawing, the section was marked with a permanent marker pen to indicate the margo anterior of the diaphysis. The section was placed in a stereomicroscope (SZ-40; Olympus, Tokyo, Japan) with the mark pointing up and was illuminated from underneath. A CCD video camera (WV-CD 130; Panasonic, Osaka, Japan) was attached to the microscope and connected to a PC (ProLinea 4/33) equipped with a frame-grabber card (LIFEVIEW; Animation Technologies, Inc., Taipei, Taiwan). Images of the sections were captured and transformed into black-and-white images by threshold filtering. An image of a reference was captured at the same magnification to convert pixel coordinates to physical coordinates. Bone area, marrow area, tissue area, axial moment of inertia, and the distance from the bending axis to the upper surface (margo anterior) of bone were measured with specially (in-house)-developed software. The axial monemt of inertia (I_x) was determined around the mediolateral axis of bending, which contains the center of mass of the 200-μm-thick section. Data presented include bone area (mm^2), marrow area (mm^2), and tissue area (mm^2; which is identical to bone area + marrow area).

Table 1. Final body weight and femur length (mean ± SD)

Groups	Hypo-zinc	Control	Hyper-zinc
Final body weight (g)	108.1 ± 13.1	218.3 ± 13.6[a]	276.4 ± 16.5[a,b]
Femur length (mm)	27.22 ± 1.02	29.58 ± 0.83[a]	32.96 ± 0.83[a,b]

[a]Significantly different from the hypo-zinc group.
[b]Significantly different from the control group.

Statistical Analysis

Because data were not normally distributed, differences between groups were evaluated with the Kruskal–Wallis test involving a multiple comparison procedure.[8] The measured femoral lengths and the weights of the rats were tested for differences between groups by analysis of variance (ANOVA) with a Bonferroni post hoc test.[1,5] Differences were considered significant at $p < 0.05$ for the Kruskal–Wallis test and $p < 0.01$ for the Bonferroni test.[5] Unless otherwise stated, results are presented as means ± standard deviations.

Results

Animal Body Weight

At time of killing, the mean body weight of the animals in group 1 was significantly lower than that of the animals in groups 2 ($p < 0.0001$) and 3 ($p < 0.0001$) (**Table 1**). The rats in group 1 also showed other signs of zinc deficiency, such as dermal lesions on the extremities, the tail, and particularly around the eyes. The mean body weight of the animals in group 3 was significantly ($p < 0.0001$) higher than that of the animals in group 2.

Femoral Length

The mean femoral length of the animals in group 1 was significantly lower than the mean femoral length of the animals in groups 2 ($p < 0.0001$) and 3 ($p < 0.0001$). Furthermore, the mean femoral length of the animals in group 3 was significantly ($p < 0.0001$) higher than that of the animals in group 2.

Femoral Diaphyseal Areas

The bone area, marrow area, and tissue area of the femoral middiaphysis are presented in **Figure 1**.

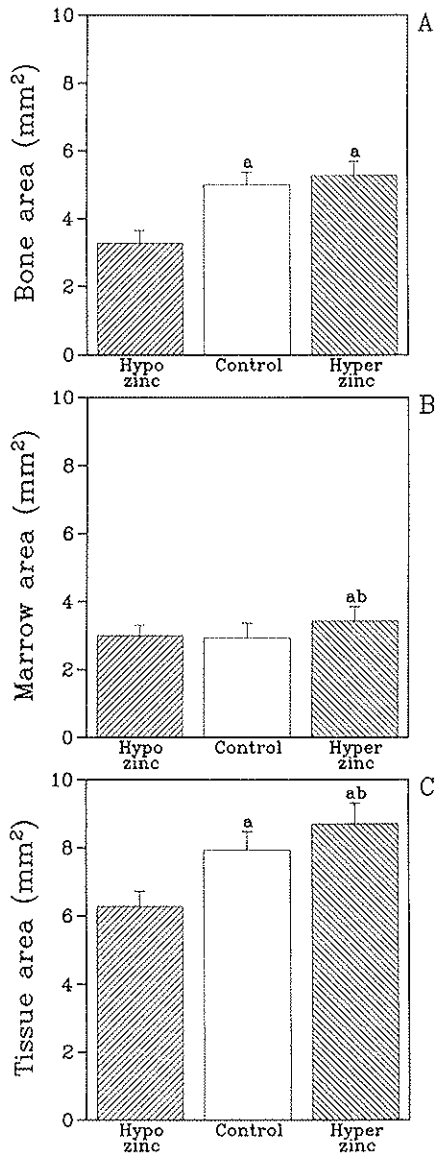

Figure 1. Femoral diaphysis area. (A) Bone area; (B) marrow area; and (C) tissue area. [a]Significantly different from the hypo-zinc group; [b]significantly different from the control group (Mean ± SD).

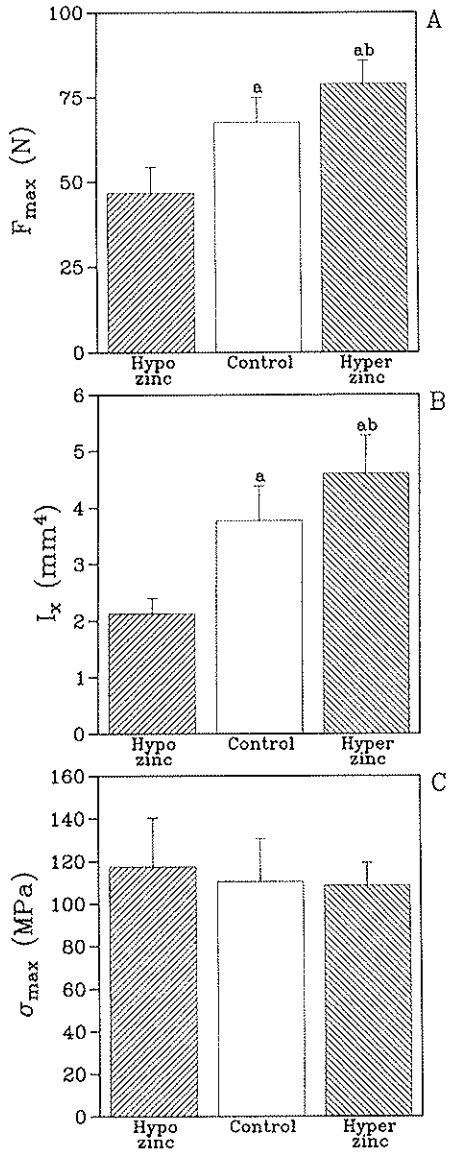

Figure 2. Femoral diaphysis. (A) Maximum load values; (B) axial moment of inertia; and (C) maximum stress values. [a]Significantly different from the hypo-zinc group; [b]significantly different from the control group (Mean ± SD).

The bone area of the femoral middiaphysis was significantly larger in group 2 ($p < 0.0001$) and group 3 ($p < 0.0001$) than in group 1. There was no significant difference between groups 2 and 3 in the diaphyseal bone area.

The marrow area was significantly larger in group 3 than in groups 1 ($p < 0.05$) and 2 ($p < 0.01$). There was no significant difference between the marrow area between groups 1 and 2. The tissue area of the femoral diaphysis was significantly larger in groups 2 ($p < 0.0001$) and 3 ($p < 0.0001$) than in group 1. Furthermore, the tissue area of group 3 was also significantly ($p < 0.005$) larger than the tissue area of group 2.

Three Point Bending Test of the Femoral Diaphysis

The results of the three point bending test of the femoral diaphysis are shown in **Figure 2**.

The maximum load values of the femoral diaphysis in groups 2 and 3 were significantly ($p < 0.0001$) higher than the maximum load values of diaphyses from group 1. The maximum load values of group 3 were also significantly ($p < 0.0005$) higher than the maximum load values of group 2.

The axial moment of inertia showed the same dose-response pattern as the maximum load values; there were significant ($p < 0.0001$) differences between groups 2 and 3 compared with group 1, and also significant ($p < 0.0005$) differences between group 2 and 3. The maximum stress values, however, showed no differences between the groups.

Femoral Neck

All femora fractured with a transcervical fracture close to the diaphysis. The results of biomechanical testing is shown in **Figure 3**.

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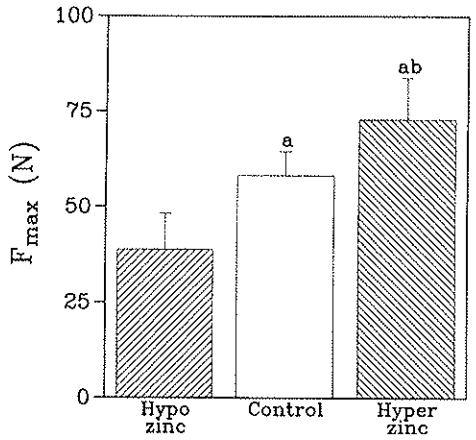

Figure 3. Femoral neck. Maximum load values. Key: [a]significantly different from the hypo-zinc group; [b]significantly different from the control group (Mean ± SD).

The maximum load values of the femoral neck were significantly ($p < 0.0001$) higher in both groups 2 and 3 compared with group 1. The maximum load values of group 3 were also significantly higher than the maximum load values of group 2 ($p < 0.0005$).

Distal Femoral Metaphysis

The results from the distal femoral metaphysis are shown in **Figure 4**. The maximum load values of the femoral metaphysis were significantly ($p < 0.0001$) higher for groups 2 and 3 than in group 1. There was no significant difference between groups 2 and 3. The ash densities and the maximum stress values exhibited the same pattern as the maximum load values at the distal femoral metaphysis.

Discussion

In the present study we have shown, for the first time, that alimentary zinc deficiency in growing rats induced a reduction of bone strength in the femoral neck, the distal femoral metaphysis, and the femoral diaphysis. These findings suggest that the bone abnormalities in zinc-deficient rats are caused not only by changes in the growth plate cartilage,[19,25,26] but also by changes in the bone size (cross-sectional area). The study has also shown that zinc supplementation has a positive effect on skeletal strength, with a clear dose-response pattern. Static histomorphometry revealed that the effects of zinc were exerted on the periosteal envelopes, mainly causing increased bone area, tissue area, and axial moment of inertia. The effect of zinc thereby mimicked the previously described effect of growth hormone or insulin-like growth factor 1 (IGF-1).[32]

Concerning our experimental model, we used Wistar rats for the investigation for several reasons: the rat is widely used for studies on bone metabolism, both in growing animals and also in the ovariectomized rat model of postmenopausal bone loss; and it is a homogeneous population, which is readily available. However, it is often believed that the rat grows continuously throughout life, because laboratory animals fed ad libitum continue to increase their body weight for a substantial part of their lifespan. This statement is still subject to discussion, but some studies have indicated that this is not the case. The growth of the rat is rapid until about 170 days and thereafter declines markedly,[23] and in old animals there is no longer evidence of osteogenesis in the growth plate.[24] Rats do not have the same pattern of remodeling as humans; that is, they lack haversian systems, which precludes cortical remodeling, but intracortical remodeling can be seen after a period of time. Therefore, cortical bone in rats has a latent remodeling capacity, which makes it likely that the basic mechanisms of bone turnover in humans also exist in rats.[22–24] This is further corroborated by the fact that rats do have cancellous bone remodeling.[14]

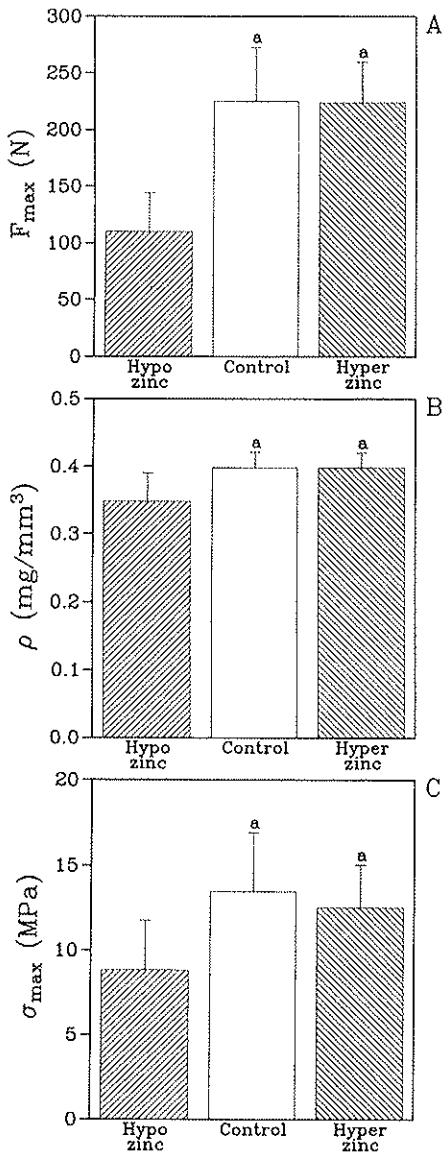

Figure 4. Distal femoral metaphysis. (A) Maximum load values; (B) ash density; and (C) maximum stress values. [a]Significantly different from the hypo-zinc group (Mean ± SD).

As noted by Eberle et al.,[12] there is a possibility that, because the rats had free access to the diets, the observed skeletal effects might be due not to zinc depletion, per se, but rather to retarded growth and reduced food intake, with subsequently lowered energy and protein intake in the zinc-deficient animals. However, as Fernandez-Madrid et al. demonstrated, by comparing zinc-deficient rats with pair-fed and ad libitum-fed controls, the impairment in protein and collagen synthesis observed in zinc-depleted rats was due to deficiency and not to differences in

caloric intake.[16] We believe that the skeletal effects of zinc deficiency found in our study were caused mainly by zinc depletion as such. Our results are supported by the findings of Bergman,[4] who compared different kinds of diet to weaning rats.

Clinically, osteoporosis is recognized as a major public health problem. Therefore, it has been of particular interest to identify anabolic agents that would increase peak bone mass and/or prevent bone loss with increasing age. Attempts to prevent osteoporosis through diet have had little success. It is of interest, however, that the daily intake of zinc is very limited in the Western world (which has a high incidence of osteoporosis).[7]

Several preclinical studies have focused on assessing the skeletal effects of different nutritional factors or pharmaceutical agents. A study by Yamaguchi et al.[44] showed that zinc and genistein (which is a natural isoflavone phytoestrogen found in leguminosae) stimulated bone formation in rats, and that a combination of zinc and genistein had a synergistic effect on bone formation. This supports the hypothesis, that the combination of nutritional factors has a potent anabolic effect on bone metabolism, which has also been demonstrated by Mühlbauer and Li,[33] who showed that a variety of salads, herbs (dried onions), and cooked vegetables increased bone formation in rats.

A few pharmaceutical agents are known to have anabolic effects on bone metabolism, among them parathyroid hormone (PTH) and growth hormone (GH).[29–31,41] Mosekilde et al.[32] showed that PTH acted mainly on the endocortical envelope in rats, whereas GH acted on the periosteal envelope, and therefore a combination of these two agents had a synergistic effect on bone strength. Vitamin D metabolites have also been shown to prevent estrogen-depletion-induced bone loss.[15] Furthermore, fluoride also has documented anabolic effects on bone mass, but in contrast to the other anabolic agents the increased bone mass during fluoride treatment does not translate into improved bone strength.[40]

In our study we investigated the effects of zinc in growing rats. We believe that zinc supplementation can improve rates of growth in weaning rats; the body weights, the lengths, and the cross-sectional areas of the femora were higher in the rats given zinc supplementation. The positive effects of zinc on bone mass in this study did not, however, seem to have a negative influence on bone quality, because the maximum stress (the maximum compressive load normalized for bone size) was unchanged in the femoral middiaphysis and increased in the distal femoral metaphysis. The unchanged maximum stress values in the diaphysis is a direct indication that tissue quality is unchanged. This in contrast to the previously mentioned animal studies with fluoride, in which an increase in bone mass did not translate into an increase in bone strength.[40] Zinc, therefore, seems to have a positive effect on the skeleton that is totally different from that of fluoride, but nearly the same as that shown for PTH, GH, and vitamin D analogs with the formation of new bone of normal biomechanical competence.[15,29–32,40,41]

Concerning GH it is known that GH secretion is, among other hormones, regulated by IGF-1 (insulin-like growth factor 1) with negative feedback. But GH also stimulates IGF-1 synthesis in the liver, and this is believed to be the active substance reaching the epiphyseal cartilage in the long bones. Some studies have shown that animals with zinc deficiency had lower serum concentrations of IGF-1 and therefore retarded growth. However, if the serum concentration of IGF-1 was restored by IGF-1 infusion to the zinc-depleted rats, this did not reverse growth retardation. These results suggest that growth retardation related to zinc deficiency cannot be explained only by the actions of IGF-1.[6,10,11,35]

If extrapolated to humans, it can be seen that zinc deficiency may impair the accumulation of peak bone mass in young children and that zinc supplementation might be beneficial.[36] A study done in Japan, in which zinc was given to short children with mild to moderate zinc deficiency, resulted in a considerable increase in height.[34]

A few studies have investigated the effects of zinc deficiency in aged rats and suggested that zinc plays a role in the pathogenesis of osteoporosis.[10,45] Concerning age-related bone loss in humans, Atik[2] found that the zinc ion levels in serum and bone tissue of patients with senile osteoporosis were lower than in those from normal patients. Two clinical studies have suggested that an increase in urinary zinc excretion may be associated with involutional osteoporosis.[2,37] Furthermore, a recent epidemiological study has shown that middle-aged and elderly men with low intakes of zinc have an increased incidence of fractures.[13]

In conclusion, under conditions of dietary deficiency of zinc the bones have poor biomechanical qualities compared with bones of control rats. Furthermore, supplemental zinc increases bone mass, size, and strength in young growing rats. There is therefore strong evidence for zinc being a very potent factor in bone metabolism, but the exact mechanism remains unknown and has to be determined. Future research should investigate whether supplemental zinc could also be beneficial for bone healing and in preventing the development of osteopenia and osteoporosis.

Acknowledgments: The authors are grateful for the excellent technical assistance of Birthe Gylling-Jørgensen, Department of Cell Biology, and Herdis B. Andersen and Thorkild A. Nielsen, Department of Neurobiology, Institute of Anatomy, University of Århus. Michael Hewitt is gratefully acknowledged for linguistic revision of the manuscript.

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Date Received: January 30, 2001
Date Revised: May 11, 2001
Date Accepted: May 30, 2001

J Musculoskel Neuron Interact 2004; 4(4):428-435

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].

The authors have no conflict of interest.

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Accepted 6 May 2004

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

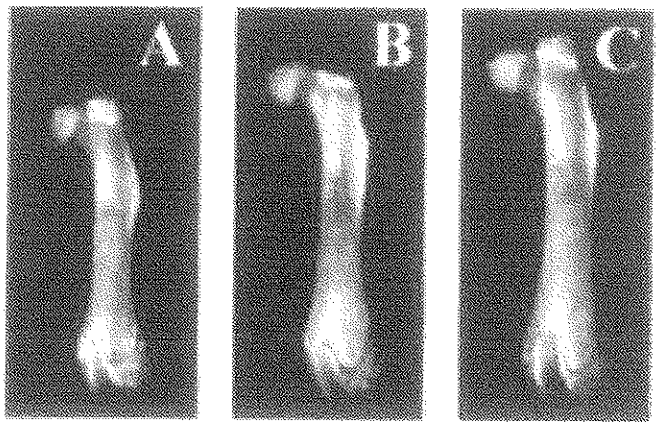

Figure 1. Femora. Group 1 (2 mg/kg) (A), group 2 (47 mg/kg) (B), and group 3 (60 mg/kg) (C).

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

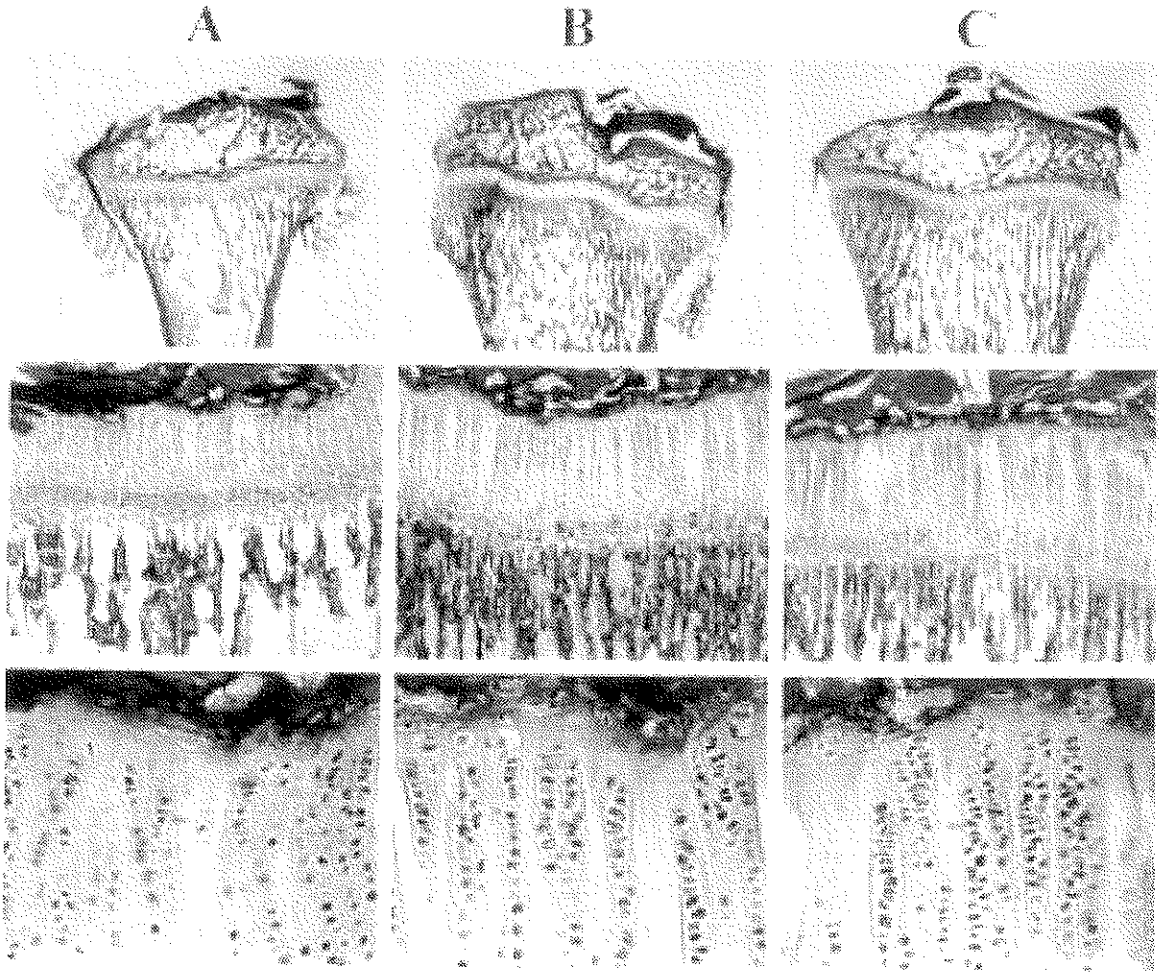

Figure 2. Goldner trichrome stained sections showing the proximale part of the tibia including the epiphyseal cartilage of the three groups at original magnifications of x12.5 (upper row), x100 (middle row), and x400 (lower row). Group 1(A), group 2 (B), and group 3 (C).

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 diecthyldithiocarbamate (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

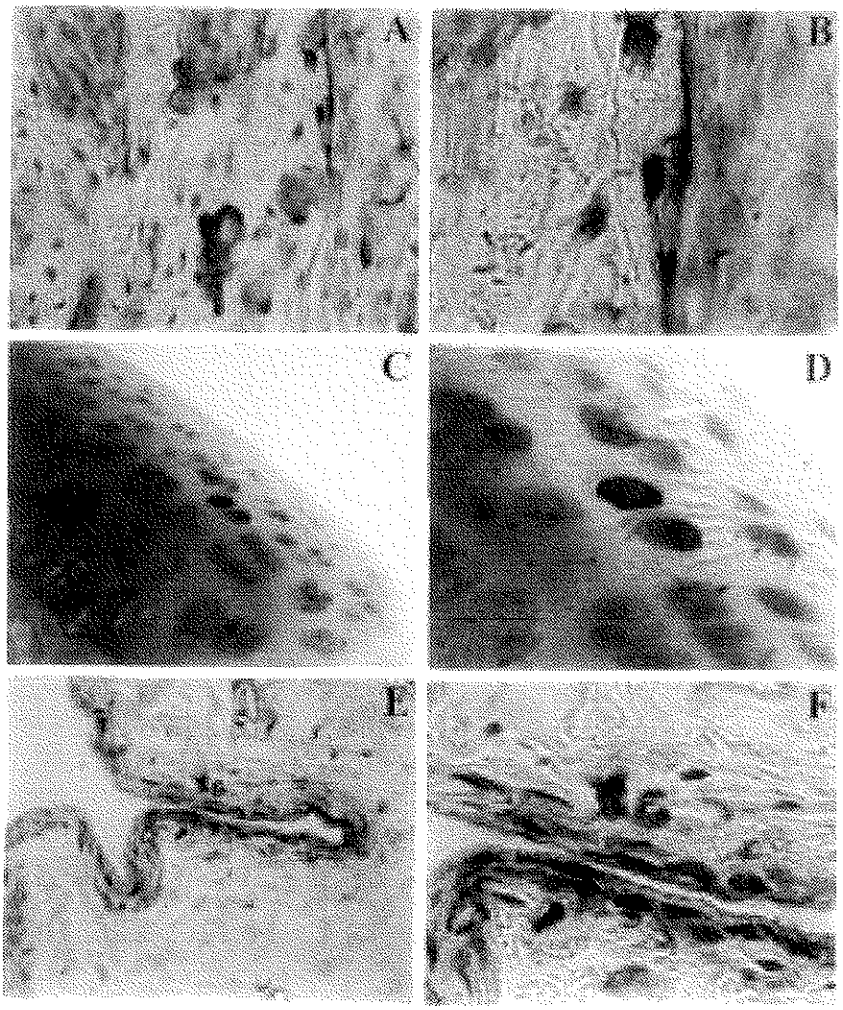

Figure 3. Toluidine blue stained sections showing the mineralizing front with osteoblasts and osteocytes. Autometallographic silver grains are seen both intracellularly and in the matrix x400 (A), osteocytes with osteocytic processes loaded with AMG grains x1000 (B). Sections showing the distal part 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

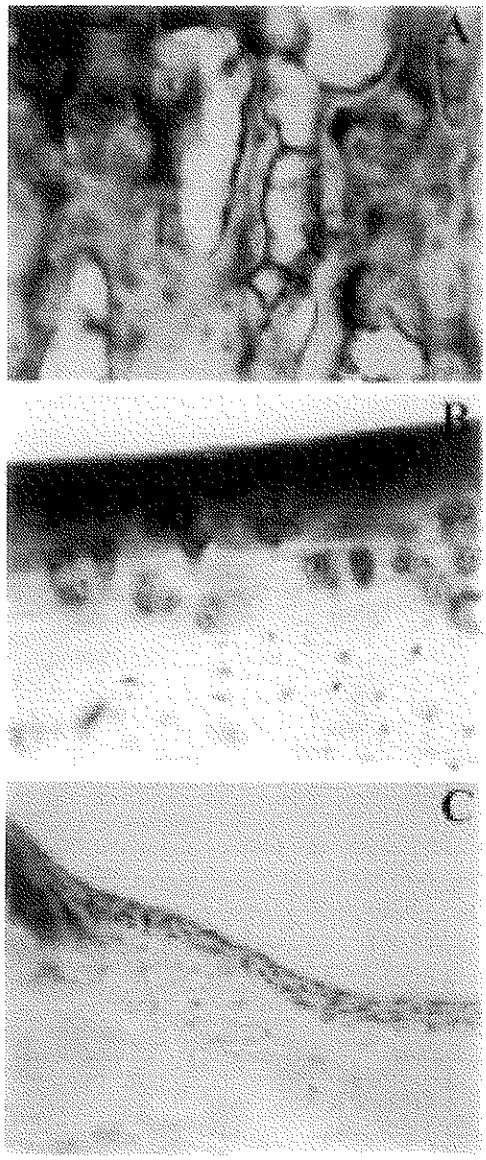

Figure 4. Toluidine blue stained sections from animals treated with diethyldithiocarbamate (DEDTC). Section showing the mineralizing front with osteoblasts and osteocytes x400 (A). Section showing the articular cartilage with chondrocytes x400 (B). Section showing the synovial membrane of the joint with 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.

Acknowledgements

The authors are grateful for the excellent technical assistance of Birthe Gylling-Jørgensen, Department of Cell Biology, and Herdis B. Andersen and Thorkild A. Nielsen, Department of Neurobiology, Institute of Anatomy, University of Århus. Karin Wiedemann is gratefully acknowledged for linguistic revision of the manuscript.

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Limited Effects of Alimentary Zinc depletion on Rat Fracture Strength and Callus Formation

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Abstract

The effects of alimentary zinc depletion on callus formation and mechanical strength of tibia fractures in rats was investigated after 3 weeks and 8 weeks of healing. The animals with zinc depletion received a diet containing 2 mg zinc per kg feed, and zinc sufficient control rats received similar feed containing 100 mg zinc per kg. The feed was given from 2 weeks before fracturing and until killing. Zinc depletion did not influence mechanical strength of the fracture after 3 weeks of healing. From 3 to 8 weeks of healing, a substantial enhancement of fracture strength was observed in both zinc sufficient animals (ultimate load: 650%, $p<0.001$; ultimate stiffness: 930%, $p<0.001$) and zinc deficient animals (ultimate load: 360%, $p<0.001$; ultimate stiffness: 850%, $p<0.001$). At 8 weeks of healing, ultimate load was decreased in the zinc deficient rats (20%, $p=0.03$), whereas ultimate stiffness did not differ (14%, $p=0.14$). After 3 weeks of healing, no differences in external callus volume, anterior-posterior diameter, or medial-lateral diameter were seen between zinc deficient and zinc sufficient animals. In the zinc sufficient groups, the external callus volume and diameters in both anterior-posterior and medial-lateral dimensions were reduced from 3 weeks to 8 weeks of healing (31%, $p<0.001$; 15%, $p<0.001$; 19%, $p<0.001$, respectively). Likewise, in the zinc deficient groups, the external callus volume and diameters in both anterior-posterior and medial-lateral dimensions were reduced from 3 weeks to 8 weeks of healing (47%, $p<0.002$; 25%, $p<0.001$; 22%, $p<0.001$, respectively). Therefore, after 8 weeks of healing, no differences in external callus volume, anterior-posterior diameter, or medial-lateral diameter were seen between zinc deficient and zinc sufficient animals. The experiment shows that zinc depletion does not influence the early callus expansion and fracture strength. During the subsequent period of remodeling zinc depletion does not influence callus reduction. However, a limited but significant decrease in fracture strength is observed.

Introduction

Zinc has been demonstrated to be an essential element for growth in humans and many animals [11, 12, 16, 28, 30, 38]. Clinically, zinc deficiency is known to be associated with retarded growth and congenital skeletal disorders [11, 12, 15, 16, 28, 30]. Many enzymes and proteins are known to contain zinc as an essential component for activity or stability. These enzymes include alkaline phosphatase, carbonic anhydrase, and deoxyribonucleic acid (DNA), all marker enzymes of bone modeling and remodeling [37, 40].

In growing rats, calcified cartilage serves as a template for bone formation (endochondral ossification). This calcification process is closely related to that seen in fracture healing. It has previously been demonstrated, that zinc administration induces an anabolic effect on skeletal strength, weight, and longitudinal growth in growing rats in a dose-dependent manner [32, 33]. Furthermore, Calhoun et al. found, that zinc is required at the site of bone formation as a requisite for complete calcification [12]. Matrix vesicles (MV) are also known to be involved in the induction of calcification of the growth plate cartilage (hypertrophic zone) [2, 19, 23, 36]. In previous studies undertaken in our laboratory it was found, that zinc is present in what is believed to be matrix vesicles [14, 32]. Another described alternative mechanism of zinc is, that zinc indirectly affect cellular activities in the growth plate through changing hormones or growth factors. Ninh et al. thus suggested, that growth retardation induced by to zinc deficiency is both related to low serum IGF-I concentrations and inhibition of the anabolic action of IGF-I [29].

In contrast to the effect of zinc on growth only little information is available about the effects of zinc on fracture healing. Fracture healing is a complex physiological cascade, which needs a number of growth factors, cytokines, and proteins around the fracture site. In vitro zinc treatment has been shown to increase some bone protein components with fracture healing [20, 21].

The aim of the present study was to determine the effects of nutritional zinc supplementation on fracture healing in vivo. A well-established animal model with standardized tibial diaphysis

fractures was used, and the influence of dietary zinc content on callus formation and mechanical strength in the rats was investigated after 3 weeks and 8 weeks of healing.

Materials and Methods

Animals and Zinc-Diets

Fracture healing was examined *in vivo* using the rat tibia fracture model as previously described in detail [3, 6]. Briefly, 80 three-month-old male Wistar rats (Møllegaard, Lille Skensved, Denmark) were randomly divided into four groups. The fractures were tested after 3 weeks and 8 weeks of healing as the callus dimensions in this model reach a maximum at 3-4 weeks of healing, whereupon the callus dimensions steadily decline as a result of remodelling [3, 6]. For each healing period two groups were tested: (1) rats that received an almost zinc-free diet containing 2 mg zinc/kg, and (2) rats given a diet containing zinc in a sufficient concentration (100 mg/kg). Both diets contained Calcium 9508 mg/kg, Phosphor 7540 mg/kg, and Vitamin D3 500.000 IE/kg (Altromin C1040; Altromin Gesellschaft für Tierernaehrung GmbH, Lage, Germany [Brogården, Gentofte, Denmark]). Consumption of these diets was initiated 2 weeks before the day of fracturing. The animals were housed with a cycle of 12 hours of light and 12 hours of darkness and had free access to tap water and diet. The rats were killed with pentobarbital (150 mg/kg body weight by intra-peritoneal injection; Mebumal, SA, Copenhagen, Denmark). The tibial bones were dissected free, and kept in buffered Ringer's Solution (4°C, pH 7.4) until mechanical testing which was performed within 6 hours.

Out of 64 rats operated on, 59 were included in the subsequent experiment (1 animal died during anaesthesia, 4 animals were killed due to a comminute fracture or a dislocated fracture). The experiment was approved by The Danish Animal Experiment Inspectorate, The Danish Ministry of Justice.

Fracture Technique

The animals were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ, USA). An unilateral, standardized closed fracture was produced above the tibiofibular junction in the

right tibia by three-point bending, and closed medullary nailing was performed using a 0.9 mm Kirschner wire [3]. The skin was closed with monofilament nylon sutures. The operations were performed under sterile conditions. Postoperatively, contact X-rays were captured to secure correct localization of the fracture and the intramedullary nail. Unprotected weight-bearing was allowed instantaneously, and the animals resumed normal activity after recovery from the anaesthesia.

Release of zinc from the Kirschner wire was measured *in-vitro*. Kirschner wires with a length similar to those used for fracture stabilisation were placed in tubes with 25 ml physiological NaCl and stored at 37°C for 8 weeks. Tubes with NaCl only served as controls. The amount of zinc in the solution was measured using an atomic spectrophotometer (Model AAnalyst 100, Perkin-Elmer, Germany).

Callus dimensions

In the fractured tibiae, the external medial-lateral and anterior-posterior diameters were measured at the fracture line with a digital sliding calliper. Total volumes of both fractured and intact tibiae were gauged using Archimedes' principle, and the external callus volume was calculated as the volume of the fractured tibia minus the volume of the intact tibia [3].

Mechanical testing

The mechanical strength of the healing fractures was measured by a destructive three-point bending procedure using a materials testing machine (Alwetron 250, Lorentzen and Wettre, Stockholm, Sweden). The intramedullary nail was removed, and the fractured bone was placed on two rounded bars (spaced 15 mm apart) with the fracture line centred between the bars. Deflection

was performed by lowering a third bar onto the fracture line, using a constant speed of 2 mm per minute. All bones were placed in the same position with the concave facet of the lateral tibial surface resting on the supporting bars, and with the load applied from the medial side. Load and deflection were recorded continuously by transducers coupled to measuring bridges. The signal was fed to an X-Y recorder, and the load-deflection curves obtained were read by a graphic tablet into a computer (HP9874A and HP9816S, Hewlett-Packard, Fort Collins, CO, USA), and ultimate load, ultimate stiffness, and deflection at ultimate load were determined. Ultimate load and stiffness describe the mechanical properties of the fracture as a whole anatomical unit (structural fracture strength).

Histology

In the present study, we used the AMG method to evaluate the pattern or concentration of zinc ions around the fracture site. This AMG method has previously been described in detail [14, 32]. 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 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, tibia sections including the fracture were cut on a diamond precision-parallel saw (Exakt; Apparatebau, Otto Hermann, Norderstedt, Germany). The sections were dipped in a 0.5% galantine solution and AMG-developed for 60–90 minutes. One animal from each group was used as control for the specificity of the autometallographic sulfide silver method. These animals were treated intaperitoneally with dieethyldithiocarbamate (DEDTC), and then allowed to live for one hour before they were perfused and processed as described above.

Statistical Analyses

The data were tested for normal distribution and homogeneity of variances and when these conditions were fulfilled, parametric analyses were applied (Students *t*-test); otherwise nonparametric analyses were used (Mann-Whitney's *U*-test). First, the zinc deficient group was compared with the zinc sufficient group at 3 weeks of healing and at 8 weeks of healing. Then changes in healing from 3 weeks to 8 weeks were tested within the zinc deficient groups and within the zinc sufficient groups. Statistical significance was determined as $p < 0.05$ (two-tailed).

Results

The results of the mechanical testing are given in Table 1. After 3 weeks of healing, no differences in fracture strength (ultimate load, ultimate stiffness, and deflection at ultimate load) were found between zinc deficient and zinc sufficient animals. From 3 to 8 weeks of healing, a substantial enhancement of fracture strength was observed in both zinc sufficient animals (ultimate load: 650%, $p<0.001$; ultimate stiffness: 930%, $p<0.001$) and zinc deficient animals (ultimate load: 360%, $p<0.001$; ultimate stiffness: 850%, $p<0.001$). At 8 weeks of healing ultimate load was significantly lower in the zinc deficient rats (20%, $p=0.03$), whereas ultimate stiffness did not differ significantly (14%, $p=0.14$)

External callus diameters and external callus volume at 3 weeks and 8 weeks of healing are given in Table 2. After 3 weeks of healing, no differences in external callus volume, anterior-posterior diameter, or medial-lateral diameter were seen between zinc deficient and zinc sufficient animals. In the zinc sufficient groups, the external callus volume and diameters in both anterior-posterior and medial-lateral dimensions were reduced from 3 weeks to 8 weeks of healing (31%, $p<0.001$; 15%, $p<0.001$; 19%, $p<0.001$, respectively). Likewise, in the zinc deficient groups, the external callus volume and diameters in both anterior-posterior and medial-lateral dimensions were reduced from 3 weeks to 8 weeks of healing (47%, $p<0.002$; 25%, $p<0.001$; 22%, $p<0.001$, respectively). Therefore, after 8 weeks of healing, no significant differences in external callus volume, anterior-posterior diameter, or medial-lateral diameter were seen between zinc deficient and zinc sufficient animals.

Neither after 3 weeks of healing nor after 8 weeks of healing was any difference in body weight gain found between zinc deficient and zinc sufficient animals (Table 2).

The Kirschner wires had a small but measurable release of zinc (Table 3). The discharge of zinc from each wire was approximately 0.5 μg during the 8 weeks observation period (Kirschner wire: 0.51±0.04 μg, vehicle without wire: 0.16±0.02 μg, (mean±SEM, $p=0.008$)).

Histology (4 animals from each group)

After 21 days the specimens from the zinc deficient group seemed to produce a more proliferative procallus than the zinc supplemented group. These differences could not be found after 56 days of healing. In spite of this, there was no histological difference between the corresponding procallus and callus of the two groups.

The ZnS AMG grains were concentrated in the osteoid in both groups and it was not possible to demonstrate any differences in the intensity or distribution pattern of the Zns AMG grains in the two groups. The procallus and callus was void of ZnS AMG grains.

The fracture sections from control animals that had been treated with dieethyldithiocarbamate (DEDTC) were completely devoid of silver grains.

Discussion

This experiment shows that alimentary zinc-supplementation significantly enhances the mechanical strength of healing fractures after 8 weeks compared with zinc-deficient rats. From 3 to 8 weeks of healing a substantial enhancement of callus mechanical quality was observed in both groups of rats. The remodelling of callus tissue during the same period resulted in nearly identical external callus volumes in the two groups at 8 weeks of healing. Therefore, we conclude that alimentary zinc-supplementation do not substantially enhanced fracture healing in young rats. These findings are similar to those reported by Milachowski et al. [25]. This could be explained by the fact that both the study by Milachowski et al. and the present study investigated young fractured rats, which have much higher zinc balances than old rats. Andreen and Larsson thus reported that old rats more readily go into negative zinc balance, than young rats, and that rats in negative zinc balance after fracture trauma are dependent on skeletal zinc stores to maintain homeostasis [8]. Other studies have shown that the zinc accumulation in intact bone in older rats is only approximately 10% of that seen in six weeks old rats [8, 39]. Mature rats do not absorb less zinc, than young rats, but secrete more endogenous zinc, initially by increased faecal excretion and later by marked increase in urinary zinc excretion [8, 13, 24, 39]. Even when we eliminated potential sources of zinc contamination from the environment, by housing the rats in metal-free cages, using distilled water and plastic equipments for storage of the dietary ingredients, feeding the rats zinc depleted diets 2 weeks before the operation, and found only very little zinc supply from the Kirschner wires, the rats were still able to maintain zinc homeostasis at the fracture site.

According to Williams and Mills the total amount of zinc in a weanling rat giving a diet with 12 mg zinc/kg is approximately 3.6 mg zinc [37]. One third of the total amount of zinc is located in the bone [8], which gives a total amount of approximately 1.2 mg zinc in the skeleton of a growing rat. Comparing the total amount of zinc in the skeleton from a growing rat and the amount of zinc given through the diet in our study (zinc-deficient group: Approximately 2.5 mg during 8

weeks of observation, zinc-sufficient group: Approximately 125 mg during the 8 weeks of observation) with the possible contamination from the Kirshner-wire (0.5 mg during the 8 weeks observation period), this is a very little but still measurable source of zinc contamination.
The duration of fracture healing was not influenced by the additional administration of zinc to the daily diets.

To the best of our knowledge only one in vivo study has shown stimulatory effect of zinc administration on fracture healing in rats: Igarashi et al. thus found that administration of zinc acexamate (10 mg Zn/100 mg) for 28 days caused a significant increase in calcium content, alkaline and acid phosphatases activities, and protein and DNA contents in the tissues of rats after fracture healing [22]. Apart from that study by Igarashi et al. only in vitro studies have reported stimulatory effect of zinc on fracture healing in young rats [20, 21, 40]. In a different study Igarashi and Yamaguchi demonstrated that fracture healing increases production of the 66 kDA protein molecule which is a major component, and that this elevation is enhanced by zinc treatment [20].

It has also been suggested, that zinc plays an important role in the development of osteopenia and osteoporosis [9, 15, 18, 31, 35], and that inadequate intakes of zinc are important risk factors for osteoporotic fractures [17, 25]. Ostoporotic fractures are widely recognized as a major problem of public health increasing with age. Therefore, it has been of particular interest to identify anabolic agents that are able to increase bone mass and prevent bone loss.

Several clinical studies have focused on assessing the skeletal effect of different nutritional factors [17, 40] or pharmaceutical agents [26, 27], whereas few studies have focused on factors stimulating fracture healing [3, 4, 5, 6, 7, 34].

In conclusion: under conditions of dietary supplementation of zinc in young rats there were no effects on the time of fracture healing or any histological differences, but the biomechanical quality of the bone in the healed fracture was higher in the zinc-supplemented group, than in the zinc-deficient group. Further research should investigate whether supplemental zinc could be

beneficial for bone healing in older individuals.

Acknowledgment

The authors are grateful for the excellent technical assistance of Jytte Utoft, Dorete Jensen, and Inger Vang Magnussen.

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Table 1.

Mechanical properties of fractured tibia after either 3 or 8 weeks of healing (mean ± SEM).

	Zn 100 mg/kg	Zn 2 mg/kg	*p*	Zn 100 mg/kg	Zn 2 mg/kg	*p*
Healing time (weeks)	3	3	–	8	8	–
Number of animals	15	14	–	16	14	–
Ultimate load (N)	20±2	25±2	0.10	145±7	116±11	0.03
Ultimate stiffness (N/mm)	59±10	64±12	0.78	610±36	524±43	0.14
Deflection at ultimate load (mm)	0.56±0.1	0.79±0.1	0.16	0.29±0.02	0.28±0.03	0.72

Table 2.

Dimensions and volume of fractured and contralateral intact tibia as well as body weights after 3 weeks and 8 weeks of healing (mean ± SEM).

	Zn 100 mg/kg	Zn 2 mg/kg	*p*	Zn 100 mg/kg	Zn 2 mg/kg	*p*
Healing time (weeks)	3	3	–	8	8	–
Number of animals	15	14	–	16	14	–
Fractured tibia						
External callus dimensions						
Anterior-posterior (mm)	6.8±0.2	7.3±0.3	0.16	5.8±0.2	5.5±0.2	0.22
Medial-lateral (mm)	5.6±0.1	5.6±0.1	0.78	4.5±0.1	4.4±0.2	0.50
Volume (mm^3)	211±13	252±30	0.78	146±12	133±11	0.45
Tibia volume (mm^3)	730±18	766±34	0.56	676±14	647±19	0.22
Contralateral intact tibia						
External bone dimensions at locations corresponding to callus measurements						
Anterior-posterior (mm)	2.9±0.03	2.9±0.04	0.68	3.0±0.02	2.9±0.04	0.31
Medial-lateral (mm)	2.7±0.03	2.6±0.04	0.09	2.8±0.04	2.8±0.04	0.67
Tibia volume (mm^3)	518±9	514±11	0.75	530±7	514±12	0.22
Body weight at						
Start of experiment (g)	341±2	341±3	0.95	341±2	340±3	0.31
Operation (g)	383±5	375±5	0.19	385±3	367±5	0.01
Sacrifice (g)	412±8	402±6	0.36	466±7	448±10	0.24
Body weight change during fracture healing (g)	29±5	27±2	0.98	80±4	81±6	0.93
Food consumption (g/day)				22±1	23±7	0.51

Table 3.

The release of zinc (mean ± SEM)

Sample	NaCl + K-wire (μmol Zn/l)	NaCl (μmol Zn/l)
1	0.357	0.087
2	0.234	0.132
3	0.337	0.064
Mean±SEM	0.309[a] ± 0.038	0.094 ± 0.020

Key: [a]significantly different from NaCl without K-wire.