EFFECTS OF ZINC DEFICIENCY ON LONGITUDINAL BONE GROWTH
IN JUVENILE CHICKENS AND YOUNG GROWING RATS

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Xibin Wang

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We approve the thesis of Xibin Wang.

Roland M. Leach  
The Walther H. Ott Professor in Avian Biology  
Thesis Advisor  
Chair of Committee

Gary J. Fosmire  
Associate Professor of Nutrition Science

Carol V. Gay  
Professor of Cell Biology

Sally E. Johnson  
Assistant Professor of Poultry Science

Ronald S. Kensinger  
Associate Professor of Animal Nutrition/Physiology  
Associate Director of the Intercollege Graduate Program in Nutrition
ABSTRACT

Zinc is essential for endochondral bone formation. Zinc deficiency affects the epiphyseal growth plate and thereby impairs longitudinal bone growth in humans and animals. In a zinc deficient state, the width of the growth plate is narrowed and the structure is histologically abnormal. However, the cellular and molecular mechanisms for the effects of zinc deficiency on bone formation have not been well-defined.

The first objective of this thesis was to investigate the effects of zinc deficiency on chondrocyte proliferation, differentiation and apoptosis in the epiphyseal growth plate of juvenile chickens. Newly hatched broiler chickens were fed either a low zinc (10 mg per kg diet, or 10 ppm) or a zinc adequate (68 ppm) soy protein-based purified diet. Cell proliferation in the growth plate was evaluated with BrdU labeling technique. Apoptosis was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method. The results showed that, as early as day 3 of the study, zinc deficiency significantly inhibited chondrocyte proliferation in the growth plate. Premature differentiation also occurred in chondrocytes of the proliferative zone, as indicated by immunostaining for osteonectin, a marker for chondrocyte maturation. Furthermore, zinc deficiency caused apoptosis of growth plate chondrocytes. These effects were primarily manifested in the areas remote from the blood supply. In addition, in situ hybridization showed that collagen II mRNA was uniformly decreased in the growth plate due to zinc deficiency. Immunostaining of local growth factors PTHrP and IGF-1 did not reveal any differences between zinc deficient and adequate tissues at the early stage. By day 7 of the
study, severe growth plate lesions, characterized by reduced cellularity, abnormally shaped cells and apoptotic bodies, were formed in areas remote from blood vessels. Collagen II mRNA, PTHrP and IGF-1 were greatly reduced in the lesion. However, the growth rate and food intake of zinc deficient chickens were not different from that of the controls during the 7 day experiment period. Therefore, zinc deficiency affected chondrocyte proliferation, differentiation, and apoptosis independently from long-term effects on nutrient intake.

The second objective of this thesis was to investigate the influence of maternal zinc deficiency during lactation and dietary zinc treatment after weaning on the longitudinal bone growth of young growing rats. Sprague-Dawley dams were fed either a zinc-adequate (34 ppm zinc) or a low zinc (4 ppm zinc) purified diet throughout lactation. After weaning at 22 days postpartum, pups received dietary treatment for another 16 days. Pups from zinc-sufficient dams were fed the zinc-adequate diet (Control), while pups from zinc-deficient dams were divided into 3 groups: one group received a low zinc diet (2 ppm zinc) and the other two groups were fed the zinc-adequate diet with (pair-fed) or without (ad libitum-fed) food restriction. Maternal zinc deficiency significantly affected the growth of suckling pups. At age of 21 days, the average body weight, femur length, and femur dry weight were 30.1 g, 16.0 mm, and 47.4 mg for the pups from zinc-deficient dams, respectively, and 56.5 g, 18.9 mm, and 76.3 mg for the pups from zinc-sufficient dams, respectively (p<0.05). Furthermore, dietary zinc deficiency after weaning completely prevented growth in pups from zinc-deficient dams. Growth of pair-fed animals was also severely impaired. In contrast, the growth rate of ad libitum-fed
animals was similar to that of the control animals. At the end of the study, femur length, femur dry weight, and the width of tibial growth pate were significantly lower in zinc-deficient and pair-fed rats as compared to ad libitum-fed and control animals. No histological differences were observed in growth plates between zinc-deficient and pair-fed rats. In conclusion, maternal zinc deficiency during lactation and dietary zinc restriction after weaning severely impaired the longitudinal bone growth of young growing rats. However, this effect was largely attributable to the inanition associated with zinc deficiency as the effect could be duplicated with food restriction.

In summary, zinc deficiency decreased proliferation, promoted differentiation and induced apoptosis in growth plate chondrocytes of juvenile chickens. These effects were responsible for the disrupted structure and formation of pathological lesions in the chicken growth plate. In young growing rats, maternal zinc deficiency and dietary zinc restriction also severely reduced the width of the growth plate and impaired longitudinal bone growth.
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Chapter 1

LITERATURE REVIEW

INTRODUCTION

The essentiality of zinc for the wellbeing of humans and animals has been extensively studied. Severe zinc deficiency causes retarded growth, loss of appetite, depressed immune function, dermatitis, impaired reproductive performance, skeletal abnormality, altered taste acuity and impaired wound healing (Cousins 1996). Among these symptoms, growth retardation remains one of the most prominent and consistent characteristics of a zinc deficient state. Abnormal longitudinal bone growth and development have been reported in humans (Prasad et al. 1963), young rats (Todd et al. 1934), fetal rats (daCunha Ferreira et al. 1989), chicks (O'Dell et al. 1958), pigs (Miller et al. 1968) and rhesus monkeys (Leek et al. 1988, Golub et al. 1996).

The effect of zinc deficiency on skeletal development has been linked to a decreased activity of growth plate tissue. In zinc deficient rats and chicks, growth plates were decreased in width and differed in structure when compared with those of zinc-adequate animals (Follis et al. 1941, O'Dell et al. 1958, Young et al. 1958, Westmoreland and Hoekstra 1969). Increasing amount of evidence suggests that zinc plays a critical role in both cell proliferation and cell survival (MacDonald 2000, Sunderman 1995, Truong-Tran et al. 2000). This function of zinc could be the biological basis for the dramatic effect of zinc deficiency on skeletal formation. Therefore, this review will first discuss biochemical properties of zinc, the role of zinc in growth and cell proliferation, and
influence of zinc on cell apoptosis. Then it will focus on mechanisms of longitudinal bone growth and effects of zinc deficiency on growth plate activity.

**BIOCHEMICAL PROPERTIES OF ZINC**

Zinc presents in biological systems as a divalent cation (Zn\(^{2+}\)). Very little of free Zn\(^{2+}\) exists in the cell or body fluids (Cousins 1996, Magneson et al. 1987). Zinc is mainly bound to proteins with cysteine, histidine, glutamate and aspartate residues being the major ligands (Vallee and Falchuk 1993). Amino acids such as cysteine, histidine also contribute to sequestering of free Zn\(^{2+}\). Binding of Zn\(^{2+}\) to the sulfhydryl group is believed to play an important role in protecting proteins against oxidative stress and thereby maintaining the normal protein function (O'Dell 2000, Powell 2000).

Zinc is a cofactor of more than 200 metalloenzymes (Mills 1989). The roles of zinc in these enzymes have been classified as catalytic and/or structural (Vallee and Falchuk 1993). Carbonic anhydrase, alkaline phosphatase and carboxypeptidase A are typical examples of enzymes where zinc plays a catalytic role, while in protein kinase C, zinc functions as a structural factor (Chesters 1997). Besides metalloenzymes, zinc is an essential component in many zinc finger proteins (Klug and Schwabe 1995). These proteins, such as transcription factor TFIIIA, retinoic acid and calcitriol receptors, play an important role in regulation of gene expression (Cousins 1996).

Zinc deficiency reduces activities of many zinc metalloenzymes such as alkaline phosphatase (Westmoreland 1971), carboxypeptidase A (Mills et al. 1967). Despite these findings, none of the zinc-dependent enzymes has been directly linked to the symptoms
associated with a zinc deficient state.

THE ROLE OF ZINC IN GROWTH AND CELL PROLIFERATION

Growth and food intake

Slow growth and depressed food intake are dominant features in zinc deficient animals (Chesters and Quantermann 1970, O’Dell et al. 1958, Young et al. 1958). In young rats, anorexia develops and growth ceases after feeding a low zinc diet for a few days (Chesters 1997, Whitelock 1998). However, increasing consumption of a zinc deficient diet by force-feeding results in severe illness and even death instead of restoring growth (Chesters and Quantermann 1970, Flanagan 1984). This indicates that growth retardation may result from an inherent metabolic defect (Chesters 1997).

Cell proliferation

Reduction in growth is correlated with decreased cell proliferation and DNA synthesis in zinc deficient animals (Southon et al. 1985, Williams and Chesters 1970). In vitro studies have also demonstrated that zinc restriction inhibits DNA synthesis in cell culture systems (Rubin 1972, Chesters et al. 1989). These studies use metal chelators such as ethylenediamine-tetraacetate (EDTA) or diethylenetriaminepentaacetate (DTPA) to reduce zinc availability. Specificity of zinc deprivation is shown by reversing the chelator effect with Zn$^{2+}$, but not with other metals such as Fe$^{2+}$, Ca$^{2+}$, Mg$^{2+}$. 
Mechanisms for the effect of zinc deficiency on cell proliferation have been explored in many studies. Zinc deficiency decreases the activities of thymidine kinase and DNA polymerases in animals (Duncan and Hurley 1978) and cultured cells (Chesters et al. 1990, Lieberman et al. 1963). Thymidine kinase catalyzes the phosphorylation of deoxythymidine to deoxythymidine monophosphate in the pyrimidine salvage pathway. Thymidine kinase activity increases in late G1 phase preceding DNA synthesis (Chesters et al. 1990, Lieberman et al. 1963). Zinc deficiency stops the cell cycle in late G1 phase, which coincides with decreased thymidine kinase activity (Chesters et al. 1989 and 1990). Thymidine kinase is not a zinc metalloenzyme, but studies have shown that zinc deficiency inhibits the gene expression of thymidine kinase at the transcription level (Chesters et al. 1995, Prasad et al. 1996). These data suggest that DNA synthesis could be blocked at the point of thymidine kinase. However, when cells express a transfected thymidine kinase gene, which is not influenced by zinc status, $^3$H-thymidine incorporation is still inhibited by zinc restriction (Chesters et al. 1993). This indicates that zinc is also required for other limiting steps prior to DNA synthesis.

MacDonald et al. (1998) have demonstrated that DTPA inhibits IGF-1 induced DNA synthesis. Therefore, it is postulated that zinc deficiency interferes with signaling pathways of the growth factor during late G1 phase (MacDonald 2000). Although zinc restriction slightly decreases IGF-1 binding to the receptor, it does not inhibit phosphorylation (activation) of the receptor and insulin response substrate (IRS) while still preventing DNA synthesis (MacDonald 2000). This suggests that zinc status may influence the pathway downstream of the receptor and IRS or other pathways that are
essential for entering S phase.

In summary, zinc deficiency inhibits cell proliferation and DNA synthesis. The limiting step lies within G1 phase of the cell cycle (Chesters et al. 1989). However, the mechanism is still poorly understood at the molecular level.

INFLUENCE OF ZINC ON APOPTOSIS

Apoptosis and necrosis

Two modes of cell death, apoptosis and necrosis, have been described in the literature (Duvall and Wyllie 1986). Cells undergoing apoptosis are characterized by loss of attachment to the surrounding microenvironment, shrinkage, membrane blebbing, DNA fragmentation, chromatin condensation and disassembly into membrane bound vesicles (apoptotic bodies). In the last phase of apoptosis, macrophage or adjacent cells rapidly clear the apoptotic bodies through phagocytosis. During the process, the intracellular contents do not leak into extracellular environment and, therefore, apoptosis is not accompanied by inflammatory response. This is in contrast to necrosis, which is characterized by cell swelling, plasma membrane rupture, leakage of internal material into extracellular space causing inflammation (Wyllie 1997).

A variety of stimuli can trigger apoptosis. These include DNA injury, nutrient deprivation, virus infection, cytotoxic T cell killing, cell membrane injury, and mitochondrial injury (Green and Reed 1998, Wyllie 1997). In addition, activation of tumor necrosis factor receptor or death receptor - CD95 also signals apoptosis (Ashkenazi
and Dixit 1998). A specific family of proteases – caspases are activated during apoptosis. The enzymes cleave a set of proteins such as endonuclease inhibitor and various structural proteins, leading to DNA fragmentation and cell disassembly (Thornberry and Lazebnik 1998). For example, $I^{\text{CAD}}/\text{DFF45}$ is an inhibitor of the endonuclease responsible for DNA fragmentation. Caspase-3 cleaves $I^{\text{CAD}}/\text{DFF45}$, giving arise to a free endonuclease, which in turn degrades the DNA (Liu et al. 1997, Sakahira et al. 1998). Another example is that caspases contribute to destruction of cell structures. The nuclear membrane is supported by a rigid lamina, which is formed by polymerization of intermediate filament proteins called lamins. Active caspase-6 (Mch-2) cleaves lamins, causing decomposition of lamina and collapse of the nuclear membrane (Srinivasula et al. 1996, Takahashi et al. 1996).

**Zinc and apoptosis**

Zinc deficiency has been reported to induce cell apoptosis in the small intestine and embryonic tissues of rats (Elmes 1977, Record et al. 1985, Rogers et al. 1995). It has also been shown that zinc depletion causes thymus atrophy and lymphopenia in mice (Fraker et al. 1977). Further studies have revealed that changes in the lymphocytic system are due to high loss of precursor T and B cells in the bone marrow through apoptosis (Fraker et al. 2000, King et al. 1995, Osati et al. 1998). However, this effect of zinc deficiency is mediated by increased glucocorticoid levels (DePasquale-Jardieu and Fraker 1979 and 1980).

Direct influence of zinc on apoptosis has been demonstrated in cell culture
systems. First, cells cultured in a low zinc medium have been shown to become apoptotic (Martin et al. 1991). Second, apoptosis can be induced by membrane-permeable metal chelators such as tetrakis (2-pyridyl methyl)-ethylenediamine (TPEN), or 1, 10-phenanthroline, which can be reversed by addition of Zn$^{2+}$ (McCabe et al. 1993, Zalewski et al. 1991). Third, zinc supplementation to the culture medium can also prevent apoptosis induced by other stimuli such as tumor necrosis factor or irradiation (Flieger et al. 1989, Martin and Cotter 1991).

Studies have been carried out to investigate the mechanisms involved in zinc deficiency-induced apoptosis. It has been shown that Zn$^{2+}$ directly inhibits activities of enzymes in the apoptotic pathway such as Ca$^{2+}$/Mg$^{2+}$ endonuclease (Cohen and Duke 1984, Giannakis et al. 1991), caspase-3 (Perry et al. 1997) and caspase-6 (Stennicke and Salvesen 1997, Takahashi et al. 1996). Recently, researchers compared the effects of membrane permeable metal chelator TPEN and impermeable chelator DTPA on apoptosis of hepatocytes (Nakatani et al. 2000). TPEN increased caspase-3-like activity and induced apoptosis. These effects were associated with depletion of intracellular glutathione (GSH). Addition of Zn$^{2+}$ restored the GSH level, decreased caspase-3-like activity, and hence prevented apoptosis. N-acetyl-L-cysteine, a thiol antioxidant also inhibited TPEN induced apoptosis. In contrast, DTPA did not affect GSH levels and caspase-3-like activity and did not induce apoptosis. Therefore, it has been postulated that Zn$^{2+}$ influences apoptosis possibly through regulation of cellular redox potential (Nakatani et al. 2000).

In summary, both in vivo and in vitro studies have shown that zinc status affects
cell survival. However, the effect of zinc deficiency on apoptosis in different tissues of animals has not been thoroughly investigated.

**MECHANISMS OF LONGITUDINAL BONE GROWTH**

**The structure of the epiphyseal growth plate**

The epiphyseal growth plate is responsible for longitudinal bone growth. Chondrocytes in the growth plate form vertical columns, in which cells proximal to the end of the long bone proliferate and those distal from the end of the bone mature and hypertrophy (Figure 1). The proliferation, maturation and hypertrophy of chondrocytes are laterally coordinated so that the growth plate can be divided into distinct morphological zones: proliferative, prehypertrophic and hypertrophic zones (Howlett 1979, Hunziker 1994). In the longitudinal sections of the growth plate, chondrocytes of the proliferative zone have a flat shape and those of the prehypertrophic zone gradually grow larger and reach a full size in the hypertrophic zone.

The mammalian growth plate is a totally avascular, while the avian growth plate is poorly vascularized (Hunziker 1994). Nutrients can reach the growth plate by diffusion from epiphyseal and metaphyseal vessels (Kuettner and Pauli 1983). It has been shown that epiphyseal vessels to be the main nutritional supply for the growth plate, while metaphyseal vessels, are responsible for resorption of hypertrophic chondrocytes and formation of endochondral bones (Trueta and Amato 1960).
Figure 1: Light micrograph of growth plate tissues (proximal tibia) from a chicken (28 day-old) and a rat (38 day-old).

Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; H: Hypertrophic zone; BV: blood vessel; 2OC: secondary ossification center; EB: epiphyseal bone trabeculae; MB: metaphyseal bone trabeculae. Bar = 200mM.

Some blood vessels penetrate the chicken growth plate, while the rat growth plate is totally avascular. In the rat epiphysis, secondary ossification center expands close to the growth plate. In contrast, secondary ossification center in the chicken epiphysis has not yet extended to the growth plate.
Gene expression during chondrocyte proliferation and maturation

Longitudinal bone growth is achieved through continuous proliferation and maturation of chondrocytes and subsequent replacement with trabecular bone. During this process, gene expression is changed in chondrocytes (Pines et al. 1998, Poole et al. 1989). For example, type II collagen is mainly synthesized by proliferative chondrocytes, whereas type X collagen, osteonectin, osteopontin, bone sialoprotein and alkaline phosphatase are expressed during chondrocyte maturation and hypertrophy (Bianco et al. 1991, Knopov et al. 1995, Kwan et al. 1989, Pines et al. 1998, Poole et al. 1989, Wu et al. 1996). These gene products have been used as markers to evaluate the stage of chondrocyte differentiation. Disruption of the gene expression pattern is involved in disorders of longitudinal bone growth (Francomano et al. 1996, Pines et al. 1998).

Growth factors involved in longitudinal bone growth

The endochondral bone formation is influenced by systemic and local growth factors such as growth hormone (GH), insulin-like growth factors (IGF), parathyroid hormone related protein (PTHrP) and fibroblast growth factors (FGF). The function of these growth factors is briefly discussed in the following.

1. **GH and IGF-1.** GH is one of the most important systemic factors influencing linear growth. Studies have shown two effects of growth hormone on the epiphyseal growth plate. One is a direct effect: GH stimulates the slowly dividing prechondrocytes in the germinative cell layer of the growth plates (Ohlsson et al. 1992). The other one is an indirect effect: GH stimulates IGF-I secretion from liver or local expression in the growth

2. PTHrP. A series of gene knockout and overexpression studies have shown that PTHrP regulate the process of chondrocyte proliferation and differentiation (Stevens and Williams 1999). PTHrP knockout mice show reduced chondrocyte proliferation, premature hypertrophy and advanced endochondral ossification in the growth plates (Amizuka et al. 1994, Karaplis et al. 1994), while transgenic mice with over-expression of PTHrP display an opposite phenotype: an elongated proliferative zone and prehypertrophic zone with delayed endochondral ossification (Weir et al. 1996). Knockout of the PTH/PTHrP receptor has similar effects on the growth plate as shown in the PTHrP depleted mutants (Lanske et al. 1996). In contrast, overexpression of a constitutively activated PTH/PTHrP receptor can compensate for the loss of PTHrP (Schipani et al. 1997). Therefore, PTHrP and PTH/PTHrP receptor play an important role in endochondral bone formation.

Earlier studies have demonstrated that PTHrP is expressed in periarticular perichondrium during embryonic development, while PTH/PTHrP receptor is located on prehypertrophic chondrocytes (Lee et al. 1995, Vortkamp et al. 1996). It has been postulated that PTHrP diffuses to its receptor acting as a paracrine factor (Vortkamp et al.
However, Medill et al. (2000) have recently detected PTHrP mRNA with RT-PCR in both proliferative and mature chondrocytes, suggesting that PTHrP is produced in the growth plate and functions as an autocrine factor.

Studies have demonstrated that PTHrP production is regulated by a signaling molecule Indian hedgehog (Ihh). Overexpression of Ihh increases PTHrP production, while Ihh null mutants show a diminished PTHrP expression (St-Jacques et al. 1999, Vortkamp et al. 1996). Although the signaling pathway through which Ihh regulates PTHrP is not clear, studies have suggested that zinc finger genes could be involved (Mo et al. 1997, Campbell and Tomlinson 2000).

3. FGF. Fibroblast growth factors are also essential for cartilage growth. During embryonic development, FGFs initiate limb bud formation (Tanaka and Gann 1995). It has also been shown that exogenous FGF-2 stimulates limb outgrowth (Li et al. 1996). In primary cell culture system, FGFs 2, 4, 6, 8, and 9 stimulate avian chondrocyte proliferation in the presence of IGF-1 and transforming growth factor β (TGF-β), while at the same time FGFs 2, 4, 8, and 9 inhibit chondrocyte hypertrophy (Praul et al. 2001). In transgenic mice, overexpression of FGF-2 expands the proliferative zone in the growth plate (Coffin et al. 1995). Furthermore, a neonatal lethal mutation causing constitutive activation of FGF receptor-3 (FGFR3) enhances chondrocyte proliferation (Iwata et al. 2000). However, other studies have demonstrated opposite effects of FGFs and FGFR receptors. FGF-1 has been shown to inhibit chondrocyte proliferation in cultured murine growth plates (Sahni et al. 1999). Another form of constitutively active FGFR3 has been associated with the most common genetic form of dwarfism - achondroplasia (Shiang et
al. 1994, Bonaventure et al. 1996). In addition, null mutations of FGFR-3 in mouse causes skeletal overgrowth (Colvin et al. 1996, Deng et al. 1996). The conflicting results indicate FGFs may be involved in various signaling pathways, which in different systems have inhibitory or stimulatory effects on chondrocyte proliferation. Either way, FGFs play an important role in endochondral bone formation.

In summary, systemic hormones and local autocrine/paracrine growth factors coordinately regulate chondrocyte proliferation and differentiation in the growth plate. Nutrition status also affects the process of bone formation. Zinc is one of several nutrients that influence longitudinal bone growth (Leek et al. 1988, O’Dell et al. 1958, Prasad et al. 1963, Todd et al. 1934). Followed is a brief review of the influence of zinc deficiency on growth plate activity and the possible mechanisms.

**EFFECTS OF ZINC DEFICIENCY ON GROWTH PLATE ACTIVITIES**

Zinc deficiency greatly reduces growth plate width in animals (Follis et al. 1941, O’Dell et al. 1958, Young et al. 1958). It was reported that the growth plate structure was disrupted and its shearing strength reduced in zinc deficient rats, which was associated with decreased collagen synthesis (Suwarnasarn et al. 1982). In the growth plate of zinc deficient chickens, chondrocytes near the blood vessels appeared normal, while cells remote from the blood vessels had abnormal shapes and were surrounded by more extracellular matrix (Young et al. 1958, Westmoreland 1971). These findings suggested that the normal process of chondrocyte proliferation and maturation was disrupted. However, the cellular and molecular changes involved have not been thoroughly studied.
Two mechanisms could be responsible for the dramatic effects of zinc deficiency on the growth plate activity. One possibility is that zinc itself is not available for the normal cellular activity. As discussed above, zinc status influences the activity of many enzymes such as thymidine kinase and DNA polymerases (Duncan and Hurley 1978), which are essential for cell proliferation. Furthermore, zinc finger transcription factors such as Gli proteins, TFIIIA, retinoic acid receptors, vitamin D receptors all require zinc for maintaining three-dimension structure (Cousins 1996, Mo et al. 1997, Campbell and Tomlinson 2000). Removal of zinc from these proteins may cause a loss of function (Zeng et al. 1991).

An alternative mechanism is that zinc deficiency could indirectly affect the growth plate through changing hormones or peptide growth factors. Studies have shown that zinc deficiency reduces serum GH and IGF-1 levels (Dorup et al. 1991, Oner et al. 1984, Roth and Kirchgessner 1997). The latter is associated with decreased IGF-I expression in liver (McNall et al. 1995, Ninh et al. 1995). These findings raise the possibility that zinc deficiency affects longitudinal bone growth through the GH-IGF axis. However, infusion of GH and IGF-1 can not reverse the effects of zinc deficiency (Cha and Rojhani 1997 and 1998, Oner et al. 1984, Browning et al. 1998). Thus, the changes in circulating GH and IGF-1 may not be fully responsible for the defects of the zinc deficient state. However, involvement of local IGF-1 in the growth plate is not clear. Furthermore, because zinc finger genes could be involved in Ihh signaling and thereby PTHrP production (Mo et al. 1997, Campbell and Tomlinson 2000), studies are needed to clarify whether PTHrP mediates the effects of zinc deficiency.
In summary, zinc deficiency induces pathological changes in the growth plate and significantly impairs the longitudinal bone growth. Further studies are needed to investigate the underlying cellular and molecular mechanisms.

**STATEMENT OF PURPOSE**

Zinc deficiency reduces growth plate width and disrupts growth plate structure in animals (Follis et al. 1941, O'Dell et al. 1958, Suwarnasarn et al. 1982, Westmoreland 1971, Young et al. 1958). The underlying cellular and molecular mechanisms await to be defined. The study described in chapter 2 first explores effects of zinc deficiency on chondrocyte proliferation, differentiation, and apoptosis in the chicken growth plate. It is then investigated whether effects of zinc deficiency are mediated through local growth factors such as PTHrP and IGF-1.

A preliminary study described in chapter 3 explores the influence of maternal zinc deficiency and subsequent dietary zinc deficiency on the longitudinal bone growth and the growth plate activity of young growing rats.
Chapter 2

INFLUENCE OF ZINC DEFICIENCY ON CHONDROCYTE PROLIFERATION AND APOPTOSIS IN THE EPIPHYSEAL GROWTH PLATE OF JUVENILE CHICKENS

ABSTRACT

The purpose of this study was to investigate the effect of zinc deficiency on chondrocyte proliferation, differentiation, and apoptosis in the epiphyseal growth plate of juvenile chickens. Newly hatched broiler chickens were fed either a low zinc (10 mg per kg diet) or a zinc adequate (68 mg per kg diet) soy protein-based purified diet. Cell proliferation in the growth plate was evaluated with BrdU labeling technique. Apoptosis was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. The results showed that, as early as day 3, zinc deficiency significantly inhibited chondrocyte proliferation in the lower proliferative zone of the growth plate. Premature differentiation occurred in chondrocytes with blocked proliferation, as indicated by immunostaining for osteonectin, a marker for chondrocyte maturation. Furthermore, apoptotic chondrocytes were observed in the lower proliferative zone as well as the prehypertrophic zone of zinc deficient tissues. These dramatic effects of zinc deficiency primarily manifested in the areas remote from the blood supply. In contrast, in situ hybridization showed that collagen II mRNA was uniformly decreased on day 3 in the zinc deficient growth plate. Immunostaining for local growth factors PTHrP and IGF-1 did not reveal any differences between zinc deficient and adequate tissues at the early stage. By day 7, severe growth plate lesions, characterized by reduced
cellularity, abnormally shaped cells and apoptotic bodies, were formed in areas remote from blood vessels. Collagen II mRNA, PTHrP and IGF-1 were greatly reduced in the lesion. However, the growth rate and food intake of zinc deficient birds were not different from that of the controls during the 7 day experiment period. Therefore, a direct effect of zinc deficiency on cell proliferation, differentiation, and apoptosis was suggested.
INTRODUCTION

The epiphyseal growth plate is responsible for longitudinal bone growth (Hunziker 1994). Chondrocytes within the growth plate continuously proliferate, differentiate, and hypertrophy in an orderly process. The hypertrophic chondrocytes are then removed and replaced by trabecular bone. These sequential events, called endochondral bone formation, are influenced by nutritional status, hormones and peptide growth factors such as growth hormone (GH), insulin-like growth factors (IGF), and parathyroid hormone related protein (PTHrP) (LuValle and Beier 2000, Stevens and Williams 1999).

Zinc is one of the nutritional factors that are essential for endochondral bone formation. Zinc deficiency impairs longitudinal bone growth in humans as well as animals (Leek et al. 1988, O'Dell et al. 1958, Prasad et al. 1963, Todd et al. 1934). This effect has been linked to a decreased growth plate activity. Studies have demonstrated that the width of the growth plate is narrowed in zinc deficient animals (Follis et al. 1941, Young et al. 1958). In the growth plate of affected chickens, the chondrocytes near blood vessels appeared normal, while the remote cells had abnormal shapes and were decreased in numbers (Westmoreland 1971, Young et al. 1958). Although these pathological changes have been described for decades, the cellular process involved has not yet been elucidated.

Evidence is mounting that zinc plays a critical role in cell proliferation, differentiation, and cell survival (for recent reviews, see Chesters 1997, MacDonald
19

2000, Truong-Tran et al. 2000). For example, dietary zinc deficiency inhibits DNA synthesis and cell proliferation in liver, kidney, spleen and intestinal tissues (Southon et al. 1985, Williams and Chesters 1970). Zinc deficiency has also been shown to induce apoptosis of enterocytes and embryonic cells in animals (Elmes 1977, Record et al. 1985, Rogers et al. 1995). In addition, in vitro studies have demonstrated that zinc restriction affects cell differentiation (Petrie et al. 1991, Morimoto et al. 1992). Based on these findings, we hypothesized that zinc deficiency altered the process of chondrocyte maturation and induced apoptosis in the chicken growth plate. To test this hypothesis, we induced a zinc deficient state in young chickens with a soy protein-based diet. Chondrocyte proliferation in the growth plate was evaluated with BrdU labeling technique. Apoptosis was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. In addition, we investigated cell differentiation with immunostaining of osteonectin (a marker for chondrocyte maturation) and in situ hybridization of collagen II mRNA (a marker for non-hypertrophic chondrocytes). Finally, in order to investigate whether local growth factors were involved in the effect of zinc deficiency, we examined IGF-1 and PTHrP in the growth plate with immunohistochemistry.

MATERIALS AND METHODS

Animal care

Newly hatched male broiler (Avian X Avian) chicks raised at the Pennsylvania
State University Poultry Education and Research Center were used in this study. Birds were maintained under a 16h : 8h (light : dark) cycle. All animal care and surgical procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University (IACUC # 94R120D097).

Experimental design

Preliminary experiments showed that severe pathological lesions were formed in growth plates of chickens fed a zinc deficient diet for 2 weeks, which was consistent with a previous report (Westmoreland 1971). In a pilot study, growth plate histology was examined in chickens consuming a low zinc diet for 3, 5, 7, 9 and 14 days. Growth plate lesions were observed on d 5, but the severity varied among chickens. After d 7, all of chickens examined had developed severe lesions. Although there were no apparent histological changes in the growth plate on d 3, in situ staining for alkaline phosphatase activity was decreased. Therefore, in order to elucidate the early events, this study focused on the cellular and molecular changes in the zinc deficient growth plate prior to (d 3) and after (d 7) lesion formation.

Ninety-six chickens were randomly divided into three groups with 4 pens of 8 chickens per pen in each group. The chickens in one group (zinc-deficient, Zn-) were fed a soy protein-based diet, which contained (by analysis) 10 mg zinc per kg of diet (Table 1). The birds in other two dietary groups (zinc-adequate) received the same soybean-based diet but supplemented with zinc carbonate so that it contained 68 mg zinc per kg of diet. The chickens in one of zinc-adequate groups were allowed free access to
diet throughout the experiment (ad libitum-fed, ALZn+). The chickens in the other zinc-adequate group were fed ad libitum for the first four days, from the fifth day to the end of the experiment, they were given the amount of diet consumed in the previous 24 hours by their zinc-deficient counterparts (pair-fed, PFZn+). Deionized distilled water was provided ad libitum. Feed intake was recorded daily. The birds were weighed at the beginning of the experiment and before being killed.

**Tissue preparation**

Four chickens from each pen of the three treatments were killed by cervical dislocation after 3 days and 7 days of feeding. On each of these days, two birds per pen were injected intraperitoneally with 50mg of Bromodeoxyuridine (BrdU, Sigma) per kg body weight one hour prior to sacrifice. Blood was drawn from 2 birds per pen by cardiac puncture and collected in 2.7 ml monovette syringes (Sarstedt, Nümbrecht, Germany) with potassium-EDTA (1.6 mg EDTA/ml blood) as an anti-clotting factor. The blood was immediately centrifuged to separate the plasma, which was stored at -20°C for zinc analysis. The proximal tibiotarsi were dissected and either fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), or immediately immersed in isopentane (at melting point -159.9°C) which was prechilled in liquid nitrogen. The tissues fixed in 4%
### Table 1: Composition of diets fed to chickens

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Zinc-deficient diet (g/kg diet)</th>
<th>Zinc-adequate diet (g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated soy protein¹</td>
<td>220.0</td>
<td>220.0</td>
</tr>
<tr>
<td>Sucrose²</td>
<td>150.0</td>
<td>150.0</td>
</tr>
<tr>
<td>Glucose monohydrate³</td>
<td>477.7</td>
<td>477.7</td>
</tr>
<tr>
<td>Egg albumen</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Corn oil⁴</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Vitamin mix⁵</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Trace mineral mix⁶</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>CaHPO₄·2H₂O</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>13.8</td>
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<td>CaCO₃</td>
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</tr>
<tr>
<td>NaCl</td>
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<td>6.0</td>
</tr>
<tr>
<td>ZnCO₃⁷</td>
<td>-</td>
<td>0.115</td>
</tr>
</tbody>
</table>

¹Purina assay protein, RP-100 Ralson Purina Co. MO.

²Domino extra-fine granules, Amstar Co., New York, NY.

³Cerelose, Corn products, Englewood Cliffs, NJ.

⁴Mazola corn oil, Best Foods, Englewood Cliffs, NJ.

⁵Supplies (per kg of diet): inositol, 0.250 g; thiamin HCl, 0.011 g; riboflavin, 0.011 g; pyridoxine HCl, 0.0045 g; niacin, 0.050 g; calcium pantothenate, 0.020 g; folic acid, 0.004 g; biotin, 0.0002 g; vitamin B₁₂, 20 μg; vitamin A, 5400 IU; vitamin E, 0.066 g; vitamin D₃, 980 IU; menadione sodium bisulfite, 0.00152; choline chloride, 2.20 g; sucrose diluent.

⁶This zinc-free mineral mix supplies (per kg of diet): FeSO₄·7H₂O, 0.33 g; MgSO₄, 2.5 g; KI, 0.0026 g; CuSO₄·5H₂O, 0.0167 g; MnSO₄·H₂O, 0.333 g; CoCl₂·6H₂O, 0.0017 g; NaMoO₄·2H₂O, 0.0083 g; NaSeO₃·5H₂O, 0.0033 g; CrK(SO₄)₂·12H₂O, 0.020 g; sucrose diluent.

⁷Zinc carbonate supplies 60 mg zinc per kg diet for the zinc-adequate diet. The measured zinc concentrations for the zinc-deficient and zinc-adequate diets are 10.53 ± 0.13 and 68.67 ± 0.36 mg per kg diet, respectively (means ± SD of duplicate measurements). The zinc requirement for chickens is estimated to range from 35 to 40 mg zinc per kg diet when soybean is the main source of protein (Subcommittee on Poultry Nutrition et al. 1994).
paraformaldehyde were embedded in paraffin and serial 5 µm-thick longitudinal sections were prepared and stained with hematoxin-eosin (H & E). Unstained sections were used for immunohistochemistry and in situ hybridization. Frozen tissues were mounted in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) on dry ice. Serial, eight µm-thick longitudinal and cross sections of frozen tissues were cut on a cryostat (Richard-Allan Scientific, Kalamazoo, MI) and collected on Superfrost/plus microscope slides (Fisher Scientific, Pittsburgh, PA). Frozen sections were used for examination of alkaline phosphatase activity, nuclear BrdU incorporation and in situ apoptotic cell labeling. Schematic diagram of longitudinal and cross sections of a growth plate is shown in Figure 2.

Definitions

Because the histological zones in a growth plate have been described with different nomenclature in the literature (Howlett 1979, Hunziker 1994, Twal et al. 1994, Westmoreland 1971), the terms used in this thesis are defined as follows (Figure 2B):

1. Proliferative zone: The proliferative zone consists of chondrocytes, which are flattened and in cell proliferation cycles. One of the hallmarks of cell proliferation is DNA synthesis, which can be labeled, for example, with BrdU.

2. Prehypertrophic zone: The prehypertrophic zone is composed of chondrocytes, which are flattened or semi-flattened and do not proliferate.

3. Hypertrophic zone: The hypertrophic zone is composed of chondrocytes, which are enlarged and rounded.
Figure 2: Schematic diagrams of proximal tibial growth plates.
A: Schematic diagram of a growth plate in 3 dimensions. Longitudinal sections are cut along chondrocyte columns and cross sections are cut across chondrocyte columns. B: Schematic diagram of a longitudinal section of tibial growth plates. The growth plate is horizontally divided into proliferative, prehypertrophic and hypertrophic zones.
Measurement of the growth plate width

H & E stained longitudinal sections of growth plates were examined under a Nikon TMS microscope. The width of proliferative and prehypertrophic zones was measured at three different locations in one section from one chicken per pen of the three dietary groups. The width of the hypertrophic zone was not measured due to its connection with an embryonic cartilage cone in the middle of the bone during the first few days of postnatal life.

Mineral analysis

Diet samples were digested in instra-analyzed nitric acid on a hot plate (Thermolyne 2200, Thermolyne Corp., Dubuque, IA) at 100°C (Clegg et al. 1981) and plasma samples were diluted 4 times with deionized distilled water before zinc analysis. Zinc concentration was determined on a flame atomic absorption spectrophotometer (Instrumental Laboratory Video 11, Allied Analytical Systems, Andover, MA) at wavelength of 213.9nm. Zinc standard (1000 µg/ml) (Sigma) was diluted with deionized distilled water to form a series of standards which ranged from 0 µg Zn/ml to 1 µg Zn/ml.

Alkaline phosphatase activity

Frozen sections were fixed with cold acetone for 5 min and then incubated for 5 min at room temperature in a medium containing 2 mmol/L α-naphthyl acid phosphate (Sigma Chemical Co., St. Louis, MO), 2 mmol/L magnesium chloride and 1mg/ml Fast
Blue RR (Sigma) in 10 mmol/L sodium borate buffer pH 9.3 (Hunt 1966, Farquharson et al. 1992b). Sections were then rinsed with 0.1 mol/L acetic acid to stop the enzyme reaction and then washed three times with distilled water (each wash 5 min). Finally, the sections were mounted with a polyvinylpyrrolidone (PVP) medium consisting of 1 g of PVP (Sigma), 1 ml of distilled water and 0.04 ml of glycerin (Hunt 1966). Sections were examined under a Nikon TMS microscope and photographed with Kodak Gold 100 speed color print film.

**BrdU incorporation**

Nuclei with incorporated BrdU were detected using a procedure described by Farquharson and Loveridge (1990). Frozen sections were fixed with cold acetone for 5 min and air-dried. Nuclear DNA was denatured with 1.5 mol/L HCl for 30 min at room temperature, followed by three washes with PBS, pH 7.4. Sections were incubated for two hours at 37°C with a monoclonal antibody (primary antibody) to BrdU (Sigma) diluted 1:25 in PBS. After washing with PBS, the sections were incubated for another hour at room temperature with a secondary antibody, goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Cat. # B2752, Molecular Probes, Inc., Eugene, OR) diluted 1:25 in PBS. The sections were then washed with PBS and stained for 5 min at room temperature with 4, 6-Diamindino-2-phenylindole (DAPI, Sigma) at a concentration of 100 μg/L. DAPI is a DNA binding reagent, which stains all of the nuclei. Finally, sections were counterstained with 0.004% Evan’s blue (Sigma) for 10 min and mounted in Fluoromount-G (Southern Biotechnology Associates, Inc.)
Birmingham, AL). Sections from BrdU labeled tissue incubated with non-immune mouse serum or non-BrdU labeled tissue incubated with the primary antibody served as negative controls. All antibody solutions were clarified by brief centrifugation before use. Sections were examined under a Nikon Eclipse TE200 fluorescence microscope. Excitation and emission wavelengths for fluorescein were 495 and 525 nm, respectively. For DAPI, excitation and emission wavelengths were 360 and 450 nm, respectively.

One section of tibiae from each BrdU-injected chicken was studied. Two fields of BrdU-labeled zone in the longitudinal section of each growth plate were photographed for BrdU and DAPI labeling with Kodak Gold 800 speed color print film. The width of BrdU-labeled zone in longitudinal sections of growth plates was measured as the distance between the two horizontal borders of the labeled zone. BrdU and DAPI labeled nuclei were counted in the same field of BrdU-labeled zone (each field of BrdU-labeled zone contained approximately 400 to 600 DAPI labeled nuclei in the control growth plates). The BrdU labeling index was calculated as the ratio of the number of BrdU labeled nuclei to the number of DAPI labeled nuclei (total nuclei) in the same field.

**In situ cell death detection**

*In situ* cell death was detected with terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) described by Darfler and Karaszkiewicz (1995). Briefly, frozen sections were fixed with 10% buffered formalin (VWR Scientific Products, West Chester, PA) for 15 min at room temperature, washed twice with PBS and 70% ethanol, and air-dried. Sections were then digested for 15 min at 37°C with
proteinase K (Sigma) at a concentration of 2 µg/ml and washed three times with PBS. Sections were incubated for 15 min at 37°C with 25 µl of a solution containing 250 U/ml terminal transferase, 50 nmol/ml fluorescein-dUTP, 2.5 mmol/L CoCl2, 0.2 mol/L potassium cacodylate, 0.25 mg/ml BSA, and 25 mmol/L Tris-HCl pH 6.6 (Boehringer Mannheim, Indianapolis, IN). After washing with PBS, sections were stained for 5 min at room temperature with DAPI at a concentration of 100 µg/L. Finally, sections were counterstained with 0.004% Evan’s blue for 10 min and mounted in Fluoromount-G. Sections from the tibiae of two birds in each dietary group were examined and photographs were taken under with fluorescence microscopy described above.

**Immunohistochemistry**

Paraffin-embedded sections were deparaffinized and rehydrated prior to immunostaining with sequential washes in the following solutions: xylene, xylene, xylene/ethanol (1:1), 100% ethanol, 95% ethanol, 70% ethanol, distilled water and PBS, each for 5 min at room temperature.

1. **Immunostaining of osteonectin.** To improve penetration of immunoglobulins into sections, proteoglycans and hyaluronic acid complexes were disrupted by applying 100 µl hyaluronidase (1mg/ml in 0.1 mol/L sodium acetate, 0.5 mol/L NaCl) to the sections for 30 minutes at 37°C. The sections were then washed three times with PBS. Nonspecific binding was minimized by incubating sections in 10% normal goat serum in PBS for 2 hours at room temperature. The sections were subsequently incubated with anti-osteonectin antibody (rabbit polyclonal antibody, NIH LF-8) in PBS (1:200 dilution)
overnight at 4°C. After washing with 0.5% goat serum (diluted in PBS), the sections were incubated with goat anti-rabbit IgG conjugated to FITC (Cat. # B-2766, Molecular Probes, Inc.) in 0.5% goat serum (1:250) for 1 hour and washed again with 0.5% goat serum. Finally, the sections were counterstained with 0.004% Evan’s blue (Sigma) for 10 min and mounted in Fluoromount-G. As controls, sections were incubated with non-immune rabbit serum (1:200) instead of the primary antibody. When not specified, steps in the procedure were carried out at room temperature. Sections were examined and photographed with fluorescence microscopy.

2. Immunostaining of PTHrP. Deparaffinized sections were pretreated with hyaluronidase (1mg/ml in 0.1 mol/L sodium acetate, 0.5 mol/L NaCl) for 30 minutes at 37°C and washed three times with PBS. The sections were immersed in 0.3% H2O2 (Sigma) for 30 minutes at room temperature and washed with PBS to eliminate endogenous peroxidase activity. Nonspecific binding was minimized by incubating sections in 10% normal goat serum in PBS for 2 hours at room temperature. The sections were then incubated overnight at 4°C with anti-PTHrP antibody (rabbit polyclonal antibody raised against residues 34-53 of human PTHrP, Calbiochem, La Jolla, California) at a concentration of 4 µg/ml in PBS. After washing with PBS, the sections were incubated with biotinylated goat anti-rabbit Ig G (Cat. # B-7389, Sigma) in PBS (1:100 dilution) for 30 minutes and washed again with PBS. The sections were then incubated in ABC reagent (Vectastain kit, Vector Laboratories, Inc., Burlingame, CA) for 20 minutes. This reagent contains avidin complexed with horseradish peroxidase, which binds to the biotinylated secondary antibody. The sections were rinsed with PBS and 1%
Triton-X 100 in PBS to remove nonspecifically bound ABC complex. The sections were subsequently incubated for 8 min with 100 µl of a solution containing 67 µg diaminobenzadine tetrahydrochloride (DAB, peroxidase substrate, Sigma) and 80 µl 30% H₂O₂. Following a rinse with distilled water, the sections were mounted with 90% glycerol in PBS. For negative controls, non-immune rabbit Ig G (4 µg/ml) (Cat. # I-5006, Sigma) was substituted for the primary antibody. When not specified, steps in the procedure were carried out at room temperature. The sections were examined under a Nikon TMS microscope and photographed with Kodak Gold 100 speed color print film.

3. Immunostaining of IGF-1. Deparaffinized sections were pretreated with hyaluronidase (1mg/ml in 0.1 mol/L sodium acetate, 0.5 mol/L NaCl) for 30 minutes at 37°C and washed three times with PBS. The sections were immersed in 0.3% H₂O₂ for 30 minutes at room temperature and washed with PBS to eliminate endogenous peroxidase activity. Nonspecific binding was minimized by incubating sections in 10% normal goat serum in PBS for 2 hours at room temperature. The sections were then incubated overnight at 4°C with anti-IGF-1 antibody (rabbit polyclonal antibody, NIH #UB3-189) in PBS (1:200 dilution). After washing with 0.5% goat serum (diluted in PBS), the sections were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Cat. # A6154, Sigma) in 0.5% goat serum (1:250) for 1 hour and washed again with 0.5% goat serum. The sections were subsequently incubated for 8 min with 100 µl of a solution containing 67µg diaminobenzadine tetrahydrochloride (DAB, peroxidase substrate, Sigma) and 80 µl 30% H₂O₂. Following a rinse with distilled water, the sections were mounted with 90% glycerol in PBS. For negative controls, non immune
rabbit IgG (Cat. # I-5006, Sigma) diluted in PBS (1:200) was substituted for the primary antibody. When not specified, steps in the procedure were carried out at room temperature. The sections were examined under a Nikon TMS microscope and photographed as described in the above section.

**In situ hybridization**

Collagen II mRNA in growth plates was detected with an *in situ* hybridization procedure described by Pines et al. (1998). Paraffin-embedded sections were deparaffinized in zylene, rehydrated with sequential washes in graded ethanol solutions (100%, 95%, 70%) and rinsed in DEPC- treated water. The sections were then incubated in 2X SSC (1X SSC contained 0.15 mole/L NaCl and 0.015 mole/L Na citrate) at 70°C for 30 min. After rinsing with DEPC- treated water, the sections were treated with pronase (0.125 mg/ml in 50 mmole/L Tris-HCl, 5 mmole/L EDTA, pH7.5) for 10 minutes at room temperature. After digestion, the sections were rinsed with DEPC- treated water, fixed in 10% formalin for 20 min, blocked with 0.2% glycine for 5 min, rinsed with DEPC- treated water, rapidly dehydrated through graded ethanol solutions (70%, 95%, 100%) and air-dried for several hours. The sections were then pre-hybridized for one hour at 42°C in 100 µl of hybridization solution consisting of 50% formamide (Fisher Scientific, Fair-lawn, NJ), 10% dextran sulfate (Fisher Scientific), 1X Denhardt’s (Sigma), 250 (g/ml of yeast total RNA (Sigma), 10 mmole/L dithiothreitol (Sigma), 5mmole/L EDTA, 0.3 mole/L NaCl, 10 mmole/L Tris-HCl (diluted from 0.2 mole/L stock, pH7.5) and 10 mmole/L Na₂HPO₄- NaH₂PO₄ (diluted from 0.2 mole/L stock,
pH6.8). The sections were then hybridized overnight at 42°C with one million counts of labeled probe (described below) diluted to 100 µl with the hybridization solution. During pre-hybridization and hybridization, solutions were sealed in Coverwell (Grace Bio-labs, Bend, OR) to prevent evaporation. After hybridization, the sections were washed in 2X SSC, 1X SSC, and 0.1X SSC each for 30 minutes at 42°C. Next, the sections were dehydrated through graded ethanol, air-dried and dipped in Kodak NTB2 emulsion (Kodak, Rochester, NY). The sections were kept in a light-free, dry chamber for several days. Autoradiography was developed with sequential incubation of the sections in Kodak D-19 developer and fixer solutions, each for 5 min.

An 800 kb BamHI-PstI fragment of pDLRII containing chicken collagen II cDNA was isolated by agarose gel electrophoresis followed by electro-elution with an Elutrap (Schleicher & Schuel, Keene, NH). Plasmid pDLRII was provided by Dr. Phoebe Leboy of the University of Pennsylvania School of Dental Medicine (Young et al. 1984). Restriction fragments of collagen II cDNA were labeled with α-35S-dCTP (ICN Biomedicals, Inc., Costa Mesa, CA) using ready-to-go DNA labeling beads (dCTP) according to the manufacturer’s instructions (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The labeled probe was separated from free α-35S-dCTP with a Quick Spin Column (G-50 Sephadex for radiolabeled DNA purification, Boehringer Mannheim Corporation, Indianapolis, IN).

**Statistical analyses**

The experimental design was a two factor spit-plot design. Experimental units
(pens of chickens) were randomly assigned to three dietary levels and each experimental unit was divided into two subunits, randomly assigned to two levels of a time factor (after 3 days of feeding and after 7 days of feeding). Analysis of variance was conducted using a general linear model program of Minitab Statistical Software (Minitab Inc., State College, PA). When the main effect was significant, Tukey’s test was used to separate treatment means. Differences were considered to be significant at a level of p<0.05.

RESULTS

Feed intake, body weight and plasma zinc levels

Feed intake, body weight and plasma zinc concentration are shown in Figure 3. Neither feed intake nor body weight was different among chickens in zinc deficient (Zn-), pair-fed (PFZn+) and ad libitum-fed (ALZn+) groups throughout the experiment. However, the plasma zinc concentration in Zn- chickens was only 35% of that in PFZn+ and ALZn+ chickens on 3 d or 7 d (p<0.05). No differences were found between PFZn+ and ALZn+ treatments.
Figure 3: Influence of dietary zinc levels on feed intake, body weight, and plasma zinc concentration of chickens.

A: feed intake. B: body weight. C: plasma zinc concentration. Chickens were fed a low zinc (Zn-) diet or a zinc-adequate diet with (pair-fed, PFZn+) or without (ad libitum, ALZn+) food restriction. Each treatment had four pens of chickens. Each point represents the mean ± SEM (n=4). *The mean of Zn- group was significantly lower than the means of ALZn+ and PFZn+ groups on the same day (p<0.05).
Histology of growth plates

H & E staining of longitudinal sections of proximal tibiae is shown in Figure 4 (d 3) and Figure 5 (d 7). No differences in histology were observed among growth plates from PFZn+ and ALZn+ chickens on both 3 d and 7 d. Therefore, PFZn+ and ALZn+ groups are described as one zinc-adequate group (Zn+) when they were not different. H & E staining did not reveal any histological changes in growth plates of Zn- chickens on 3 d when compared to that of Zn+ birds (Figure 4). However, Zn- growth plates developed characteristic histological changes on 7 d similar to that reported by Westmoreland (1971). Firstly, the columnar structure of chondrocytes was disrupted in the growth plate (Figure 5B and 5D). Secondly, proliferative and prehypertrophic cells in areas away from growth plate-penetrating blood vessels had a variety of shapes, forming distinct histological lesions. Cellular density in those lesions was reduced and, therefore, it seemed that cells were surrounded by larger amounts of extracellular matrix. In contrast, chondrocytes in the upper proliferative zone and those in the vicinity of blood vessels appeared normal. The histological lesions extended from the prehypertrophic zone into the hypertrophic zone, where chondrocytes did not hypertrophy normally.

The width of proliferative (P) and prehypertrophic (PH) zones is shown in Figure 6. Although zinc deficiency did not influence the width of P and PH zones on 3 d, it significantly reduced the width more than 30% on d 7. From 3 d to 7 d, the width of P and PH zones increased about 50% in Zn+ growth plates, while it did not change over the same time period in Zn- growth plates.
Figure 4: Longitudinal sections of tibial growth plates (H & E stained) from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days.

The two arrows at the right side of A and B mark the top and bottom borders of the P and PH zones. The distance between the two arrows is the width of P and PH zones. Bar = 100mM. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; H: Hypertrophic zone; BV: blood vessel.)

Zn+ (A. 100 X and C. 400 X): The chondrocytes were organized in columnar structure. Some blood vessels penetrated the growth plate.

Zn- (B. 100 X and D. 400 X): No obvious histological changes were found at this stage when compared to Zn+ growth plates.
Figure 5: Longitudinal sections of tibial growth plates (H & E stained) from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 7 days.

The two arrows at the right side of A and B mark the top and bottom borders of the P and PH zones. The distance between the two arrows is the width of P and PH zones. Bar = 100mM. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; H: Hypertrophic zone; BV: blood vessel; N: Normal appearing area; L: Lesion area.)

Zn+ (A. 100 X and C. 400 X): The histology of growth plates was similar to that of day 3 (Figure 4), but the width of P and PH zones was increased.

Zn- (B. 100 X and D. 400 X): The columnar structure was disrupted in the growth plate. In areas in the vicinity of BV (N), cells had the normal flat shape, while in the area remote from BV (L), cells had a variety of shapes and were surrounded by a large amount of matrix.
Figure 6: Effect of dietary zinc levels on the width of proliferative (P) and prehypertrophic (PH) zones in tibial growth plates of chickens.

Chickens were fed a low zinc (Zn-) diet and a zinc-adequate diet with (pair-fed, PFZn+) or without (ad libitum, ALZn+) food restriction. Each treatment had four pens of chickens. One longitudinal section of a representative tibia from each pen was examined. The width of P and PH zones was illustrated in Figures 4 and Figure 5 measured according to the methods described in the section of materials and methods. Each bar represents the mean ± SEM (n =4). *The mean of Zn- group was significantly lower than the means of ALZn + and PFZn+ groups on d 7 (p < 0.05). †The widths of P and PH zones in ALZn + and PFZn+ groups were greater on d 7 than on d 3 (p<0.05).
**Alkaline phosphatase activity**

In longitudinal sections of Zn+ growth plates on d 3 and d 7, alkaline phosphatase activity was found in the late prehypertrophic and hypertrophic zones (Figure 7A and 7C). In Zn- growth plates, alkaline phosphatase staining was irregular and greatly reduced in areas away from blood vessels on both 3 d and 7 d (Figure 7B and 7D).

**BrdU labeling**

BrdU was used to label the nuclei of chondrocytes undergoing DNA synthesis in the S phase of a cell proliferation cycle. A distinct zone adjacent to articular cartilage was visualized with heavily labeling in the longitudinal sections of Zn+ growth plates (Figure 8A and Figure 9A).

Zinc deficiency greatly reduced BrdU labeled areas in the growth plate as early as d 3 (Figure 8C). In the upper proliferative zone, only a few layers of chondrocytes were labeled horizontally across the growth plate (defined as BrdU labeled zone in Zn- samples). In the lower proliferative zone, only chondrocytes in vicinity of blood vessels were positive, while the vast majority of cells stopped proliferating. Similar labeling pattern was observed on d 7 (Figure 9).

The width of BrdU labeled zone and the labeling index are shown in Figure 10. Zinc deficiency dramatically reduced the width of BrdU labeled zone by 80% on both 3 d and 7 d. In contrast, BrdU labeling index was less affected. On 3 d, the index in Zn- growth plates (18.9%) was slightly lower than that in Zn+ growth plates (22.6%, p<0.05); whereas no differences were found between Zn+ and Zn- treatments on d 7.
Figure 7: Alkaline phosphatase activity in longitudinal sections of tibial growth plates of chickens.

Chickens were fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days (A and B) and 7 days (C and D). Magnification 100X. (PH: prehypertrophic zone; H: Hypertrophic zone; BV: blood vessel.)

Zn+ (A and C): Alkaline phosphatase activity (black) was present in the prehypertrophic and hypertrophic zones of growth plates.

Zn- (B and C): Alkaline phosphatase activity was greatly decreased in areas away from blood vessels (asterisks *).
Figure 8: BrdU labeling in longitudinal sections of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100 mM. Magnification 200X. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; BV: blood vessel.)

A and C show fluorescent BrdU labeled nuclei. B and D show DAPI labeled nuclei in the same field of A and C, respectively. The two arrows at the right side of A and C mark the top and bottom borders of the BrdU labeled zone, respectively. The distance between the two arrows is the width of the BrdU labeled zone. Note that in Zn- growth plates (C), BrdU only labeled the chondrocytes of the upper P zone and those in the vicinity of the BV of the lower P zone.
Figure 9: BrdU labeling in longitudinal sections of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 7 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; BV: blood vessel.)

A and C show fluorescent BrdU labeled nuclei. B and D show DAPI labeled nuclei in the same field of A and C, respectively. The two arrows at the right side of A and C mark the top and bottom borders of the BrdU labeled zone, respectively. The distance between the two arrows is the width of the BrdU labeled zone. Similar to the finding with day 3 samples (Figure 8), BrdU only labeled the chondrocytes of the upper P zone in Zn- growth plates (C) and those in the vicinity of the BV of the lower P zone. Note that in the area away from the BV (D), nuclear density was greatly reduced.
Figure 10: Effect of dietary zinc levels on the width of BrdU labeled zone (A) and BrdU labeling index (B) in tibial growth plates of chickens.

Chickens were fed a low zinc (Zn-) diet or a zinc-adequate diet with (pair-fed, PFZn+) or without (ad libitum, ALZn+) food restriction. Each treatment had four pens of chickens. Two chickens from each pen were injected with BrdU one hour prior to sacrifice on d 3 and d 7 of the study. The width of BrdU labeled zone was illustrated in Figures 8 and 9. The width and labeling index were measured according to the methods described in the section of materials and methods. Each bar represents the mean ± SEM (n =4). *The mean of Zn- group was significantly lower than the means of ALZn+ and PFZn+ groups on the same day (p<0.05).
The width of the BrdU labeled zone and labeling index were not different between ALZn+ and PFZn+ groups on d 3 or d 7 (Figure 10).

BrdU labeling in the lower proliferative zone was further examined in cross sections of growth plates. It was confirmed that proliferating chondrocytes in Zn- samples were only located near blood vessels within a distance of about 100 µm (Figure 11C: d 3 and 12C: d 7). In Zn+ growth plates, BrdU labeling did not show a blood vessel-related pattern in either longitudinal or cross sections (Figure 11A and 12A).

**Chondrocyte apoptosis**

TUNEL method was employed to detect DNA cleavage in the process of cell apoptosis. In Zn+ growth plates, no chondrocytes were found positive in each of the growth plate zones, but some cells on the blood vessel wall or immediately adjacent to blood vessels were labeled (Figure 13A: d 3 and 14A: d 7).

Even though zinc deficiency did not change the histology of the growth plate on d 3, apoptotic labeling was observed in the lower proliferative zone and prehypertrophic zone of Zn- tissues (Figure 13C). By d 7, TUNEL-positive cells were scattered in the growth plate lesion (Figure 14C), where nuclear density was greatly reduced (Figure 14D). The numbers of labeled cells in Zn- tissues varied greatly from one chicken to another. For example, in a 450 X 450 µm field of a longitudinal section, the number could range from several to one hundred between different chickens. Because of this variation, quantification of apoptotic cells was not attempted.
Figure 11: BrdU labeling in cross sections through the lower proliferative zone of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days. One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (BV: blood vessel.)

A and C show fluorescent BrdU labeled nuclei. B and D show DAPI labeled nuclei in the same field of A and C, respectively. In Zn- tissues, BrdU labeling was absent in chondrocytes remote from the BV (C).
Figure 12: BrdU labeling in cross sections through the lower proliferative zone of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 7 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (BV: blood vessel.)

A and C show fluorescent BrdU labeled cells. B and D show DAPI labeled nuclei in the same field of A and C, respectively. Similar to the finding with day 3 samples (Figure 11), in Zn- tissues, BrdU labeling was absent in chondrocytes remote from the BV (C).
Figure 13: TUNEL labeling for apoptosis in longitudinal sections of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; BV: blood vessel.)

A and C show fluorescent nuclei labeled by TUNEL method. B and D show DAPI labeled nuclei in the same field of A and C, respectively. Note that in Zn+ growth plates (A), only cells associated with the BV wall were labeled. In Zn- growth plates (C), however, apoptotic chondrocytes were scattered throughout the lower P zone and PH zone.
Figure 14: TUNEL labeling for apoptosis in longitudinal sections of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 7 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; BV: blood vessel.)

A and C show fluorescent nuclei labeled by TUNEL method. B and D show DAPI labeled nuclei in the same field of A and C, respectively. Similar to the finding with day 3 samples (Figure 13), only cells associated with the BV wall were labeled in Zn+ growth plates (A). In Zn- growth plates (C), however, apoptotic chondrocytes were scattered throughout the lower P zone and PH zone, where nuclear density was greatly reduced.
TUNEL labeling in cross sections of the lower proliferative zone of Zn- growth plates demonstrated that apoptotic chondrocytes were present in areas about 100 µm away from blood vessels (Figure 15C: d 3 and 16C: d 7), where chondrocytes did not proliferate (Figure 11C: d 3 and 12C: d 7).

Chondrocyte apoptosis was further examined in H & E stained longitudinal sections of growth plates using light microscopy. Apoptotic bodies were found in lesion areas of the prehypertrophic zone in Zn- growth plates on d 7 (Figure 17). They were composed of small rounded cytoplasmic fragments with one or more nuclear remnants. Apoptotic bodies were not observed in Zn- growth plates on d 3 or in Zn+ growth plates on both d 3 and d 7.

**Immunohistochemistry of osteonectin, PTHrP and IGF-1**

**Osteonectin.** Chondrocytes positive for osteonectin immunostaining were observed in the prehypertrophic and hypertrophic zones of Zn+ growth plates, while little staining was present in the proliferative zone (Figure 18 Zn+: d 3 and 19 Zn+: d 7). In Zn- growth plates, chondrocytes in the vicinity of blood vessels had a staining pattern similar to that in the Zn+ growth plates. However, in areas remote from blood vessels, osteonectin positive chondrocytes extended from the prehypertrophic zone into the proliferative zone (Figure 18 Zn-: d 3 and 19 Zn-: d 7).

**PTHrP.** In Zn+ growth plates, prehypertrophic and hypertrophic chondrocytes were stained for PTHrP (Figure 20A: d 3 and 20C: d 7). The staining pattern in Zn- growth plates did not differ from that of Zn+ growth plates on d 3 (Figure 20B).
Figure 15: TUNEL labeling for apoptosis in cross sections through the lower proliferative zone of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (BV: blood vessel.)

A and C show fluorescent nuclei labeled by TUNEL method. B and D show DAPI labeled nuclei in the same field of A and C, respectively. Note that in Zn+ growth plates (A), only cells associated with the BV wall were labeled in Zn+ growth plates. In Zn- growth plates (C), however, apoptotic chondrocytes were scattered in areas remote from the BV.
Figure 16: TUNEL labeling for apoptosis in cross sections through the lower proliferative
zone of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+)
diet for 7 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM.
Magnification 200X. (BV: blood vessel.)

A and C show fluorescent nuclei labeled by TUNEL method. B and D show DAPI
labeled nuclei in the same field of A and C, respectively. Similar to the finding with day
3 samples (Figure 15), only cells associated with the BV wall were labeled in Zn+ growth
plates (A). In Zn- growth plates (C), however, apoptotic chondrocytes were scattered in
areas remote from the BV.
Figure 17: Apoptotic bodies in H & E stained longitudinal sections of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 7 days. A representative field of the prehypertrophic zone is shown. Magnification 400X. Apoptotic bodies were frequently found in the lesion area of Zn- growth plates (arrows). They were characterized by small cytoplasmic fragments with one or more nuclear remnants. No apoptotic bodies were observed in Zn+ tissues.
Figure 18: Immunolocalization of osteonectin in longitudinal sections of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days.

One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; H: hypertrophic zone; BV: blood vessel)

Zn+: Chondrocytes positive for osteonectin were found in the PH and H zones. Zn-: Positive cells extended from the PH zone into the lower P zone in areas remote from the BV (arrow).
Figure 19: Immunolocalization of osteonectin in longitudinal sections of tibial growth plates from chickens fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 7 days. One representative field from Zn+ or Zn- growth plate sections is shown. Bar = 100mM. Magnification 200X. (Ar: articular cartilage; P: proliferative zone; PH: prehypertrophic zone; BV: blood vessel.)

Zn+: Chondrocytes in the PH zone were shown to be positive for osteonectin. Zn-: Positive cells extended from the PH zone into the lower P zone in areas remote from the BV (arrow).
Figure 20: Immunolocalization of PTHrP in longitudinal sections of tibial growth plates of chickens.

Chickens were fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days (A and B) and 7 days (C and D). Magnification 400X. (PH: prehypertrophic zone; H: Hypertrophic zone.)

Day 3 (A and B): PTHrP was present in the PH and H zones of the growth plate. No differences were observed between Zn+ and Zn- growth plates.

Day 7 (C and D): Staining of PTHrP in Zn+ growth plates (C) was similar to day 3 growth plates. However, in Zn- growth plate (D), many cells in the lesion area (L) of PH and H zones were negative.
However, on d 7, chondrocytes in lesion areas of Zn- growth plates showed heterogenous staining for PTHrP, i.e. some cells were positive, while many were negative (Figure 20D).

**IGF-1.** Immunostaining of IGF-1 was only found in hypertrophic chondrocytes (Figure 21A: d 3 and 21C: d 7). No differences were observed in IGF-1 staining between Zn+ and Zn- growth plates on d 3 (Figure 21A and 21B). However, some chondrocytes in lesion areas of the hypertrophic zone in Zn- growth plates were negative for IGF-1 staining on d 7 (Figure 21D).

**In situ hybridization of collagen II mRNA**

Collagen II mRNA was detected on d 3 and d 7 in chondrocytes of articular and growth plate cartilage of Zn+ tissues (Figure 22A and 22C). The signal was intensified in the proliferative and prehypertrophic zones. By d 7, collagen II mRNA was diminished in the hypertrophic zone of the growth plate (Figure 22C).

Collagen II mRNA was dispersed in Zn- tissues on d 3 without intense hybridization in the proliferative and prehypertrophic zones (Figure 22B). On d 7, the signal was greatly decreased in lesion areas of Zn- growth plates (Figure 22D).
Figure 21: Immunolocalization of IGF-1 in longitudinal sections of tibial growth plates of chickens.

Chickens were fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days (A and B) and 7 days (C and D). Magnification 400X. (PH: prehypertrophic zone; H: Hypertrophic zone.)

Day 3 (A and B): IGF-1 was present in the hypertrophic zone of the growth plate. No differences were observed between Zn+ and Zn- growth plates.

Day 7 (C and D): Staining of IGF-1 in Zn+ growth plates (C) was similar to day 3 growth plates. However, in Zn- growth plates (D), many cells in the lesion area (L) of H zone were negative.
Figure 22: In situ hybridization of collagen II mRNA in longitudinal sections of tibial growth plates of chickens.

Chickens were fed a low zinc (Zn-) or a zinc-adequate (Zn+) diet for 3 days (A and B) and 7 days (C and D). Magnification 40X. (Ar: articular cartilage; P+PH: proliferative and prehypertrophic zones; H: hypertrophic zone.)

Zn+ (A and C): Collagen II mRNA (black) was present in articular cartilage and the growth plate. The signal intensified in the proliferative and prehypertrophic zones. By d7, collagen II mRNA was diminished in the hypertrophic zone of the growth plate (C).

Zn- (B and D): On d 3, collagen II mRNA dispersed throughout the tissue without intense hybridization in the proliferative and prehypertrophic zones (B). On 7 d, the signal was greatly decreased in lesion areas of the growth plate (D: asterisks *).
DISCUSSION

Changes in chondrocyte proliferation, differentiation, and survival: the earliest responses to zinc deficiency

This study investigated the effect of zinc deficiency on chondrocyte proliferation, differentiation, and apoptosis in the epiphyseal growth plate of young chickens. After chickens consumed a low zinc diet for only 3 days, serum zinc levels were significantly decreased by 65%. Zinc deficiency completely blocked cell proliferation in areas of the growth plate remote from blood vessels. Cells in these areas were immunostained with osteonectin, which was normally present in prehypertrophic and hypertrophic chondrocytes, indicating that the process of differentiation was accelerated. Apoptotic chondrocytes were also observed on day 3 in the lower proliferative zone as well as the prehypertrophic zone of zinc deficient tissues. By day 7, severe pathological lesions, characterized by reduced cellularity, abnormally shaped cells and apoptotic bodies, were formed in areas remote from blood vessels. Although there were dramatic changes in the growth plate, the growth rate and food intake of zinc deficient chickens were not different from that of the controls during the 7 days of the experiment period.

Rapid effects of zinc deficiency on serum zinc levels, cell proliferation and apoptosis have been reported in other studies. For example, plasma zinc concentrations were significantly decreased in rats consuming a low zinc diet for 2 or 3 days (Chesters and Will 1973, Roth and Kirchgessner 1997). Williams and Chesters (1970) have shown that zinc deficiency decreases incorporation of ³H-thymidine into DNA of rat liver,
kidney and spleen as much as 50% within 5 days. Furthermore, it has been reported that maternal zinc deficiency causes excess cell death in the rat embryo in 4 days (Rogers et al. 1995).

Decreased cell proliferation and increased apoptosis occurred before any changes in food intake, but coincided with a sharp decline in plasma zinc levels. Therefore, it was very likely that changes in cellular activity were directly associated with reduced zinc availability from the blood supply. Furthermore, local growth factors PTHrP and IGF-1 were not affected on d 3, excluding the possible involvement of indirect effects through these autocrine/paracrine factors.

**Influence of the distance to blood on cellular activities in a zinc-deficient state**

Effects of zinc deficiency in the growth plate primarily manifested in areas remote from the blood supply. These effects included inhibiting cell proliferation, promoting cell differentiation, inducing apoptosis as well as decreasing alkaline phosphatase activity. The changes in cellular activities eventually resulted in formation of severe pathological lesions in the growth plate. Comparable lesions have been reported in older chickens (10 – 28 days of age) with zinc deficiency (Young et al. 1958, Westmoreland 1971) and in turkeys with deficiencies of choline or nicotinic acid (Wise et al. 1973). It has been postulated that, in a nutrient-deficient state, cells near the blood supply take up most of the available nutrient and leave little for remote cells (Westmoreland 1971, Wise et al. 1973). Furthermore, it has been demonstrated that the rate of cellular zinc uptake is increased five fold upon addition of serum to stimulate the proliferation of G1 arrested
baby hamster kidney cells (Grummt et al. 1986). Proliferating chondrocytes may also have a high requirement for zinc. Therefore, in addition to shortage of zinc in the blood supply, a high requirement for the nutrient would also contribute to the sensitivity of the growth plate to zinc deficiency.

In addition to cells near the blood vessels, the top few layers of chondrocytes in the upper proliferative zone were less affected by zinc deficiency. Since these layers are nourished by the vascular structure in the articular cartilage (Howlett et al. 1984) and articular chondrocytes are in a quiescent phase, this may allow nutrients to diffuse to the growth plate without being totally consumed. This could account for the little effect of zinc deficiency on the proliferating cells adjacent to the articular cartilage.

**An all or none response of chondrocyte proliferation to zinc deficiency**

In the zinc deficient growth plate, BrdU-labeling was limited to only a few layers of chondrocytes in the upper proliferative zone and chondrocytes near blood vessels (within a distance less than 100 µm) in the lower proliferative zone (Figures 8 and 9). Thus, the total areas of BrdU-labeled zone were significantly decreased in the zinc deficient growth plate.

In contrast, the labeling index in BrdU-labeled zone was not severely affected by zinc deficiency (Figure 10). An index of 21 - 23% was observed in the control groups, while in the zinc deficient group, the index was about 19 –20 % and only slightly lower. Although younger chickens were used in the present study, the results were in line with a BrdU-labeling index of 21 - 25% in the growth plate of 3 week-old chickens.
The significantly decreased labeling areas and the less affected index indicated that zinc deficiency either completely stopped cell proliferation (in areas away from the blood supply) or did not affect it at all (in areas near the blood supply). This is consistent with studies in other systems. Fujioka and Lieberman (1964) showed that EDTA-induced zinc deficiency inhibited thymidine incorporation in liver cells of partially hepatectomized rats. The degree of inhibition was directly proportion to the reduction in labeled nuclei. This suggested that, for a given liver cell, zinc deficiency blocked the replication of DNA either completely or not at all. As presented in this study, whether or not the proliferation of a chondrocyte was affected depended upon its distance to the blood supply.

Premature chondrocyte differentiation

In the normal growth plate, immunostaining of osteonectin was observed in the differentiated chondrocytes, while little staining was present in the proliferating cells. Therefore, osteonectin marks the event of chondrocyte differentiation. In the zinc deficient growth plate, osteonectin staining extended from the prehypertrophic zone to the lower proliferative zone in areas where proliferation was blocked, suggesting that cells in these areas started the process of differentiation. This is similar to the finding that zinc-restriction with EDTA suppresses proliferation of HL-60 cells (a promyelocytic leukemia cell line) and promotes cell differentiation (Morimoto et al. 1992). It is unknown why zinc deficiency forces cell differentiation or whether the process resembles
normal maturation. It is tempting to postulate that differentiation may help cells survive in a nutrient-deficient state. However, apoptosis occurred in chondrocytes undergoing maturation (discussed below), suggesting that differentiation could not prevent cells from dying.

**Chondrocyte apoptosis**

The present study showed that chondrocytes in the normal growth plate of chickens were not labeled with TUNEL method. This is consistent with the findings of Praul et al. (1997). However, in the zinc deficient growth plate, TUNEL positive chondrocytes were observed as early as d 3 of the study in the lower proliferative and prehypertrophic zones, but not in the hypertrophic zone, indicating that proliferative and prehypertrophic chondrocytes were vulnerable to zinc restriction, while the terminally differentiated chondrocytes were resistant.

The results showed that proliferating and apoptotic chondrocytes were present in mutually exclusive areas in the lower proliferative zone (Figure 11, 12, 15, 16). This indicated that at least in some cells, apoptosis was initiated following blockage of proliferation. It has been demonstrated that cells cultured in a zinc deficient medium lose the proliferation capacity and become apoptotic (Martin and Cotter 1991). In vivo studies have also revealed that zinc deficiency induces apoptosis particularly in cells undergoing rapid cell division (Elmes 1977, Record et al. 1985). As discussed above, the proliferating cells may have a higher requirement for zinc. However, another question arises as to why proliferating cells do not arrest in a quiescent phase instead of
‘committing suicide’.

One possibility is that a low level of zinc, inhibiting cell proliferation, is sufficient to trigger apoptosis. It is conceivable that zinc deficiency could affect totally unrelated pathways at the same time. On one hand, zinc restriction may decrease the activities of thymidine kinase and DNA polymerase (Duncan and Hurley 1978) and impair cell commitment into S phase (Chesters et al. 1989). On the other hand, a lowered zinc level could initiate the process of apoptosis by reducing the inhibitory effect on Ca^{2+}/Mg^{2+}-dependent endonuclease (Giannakis et al. 1991) and caspases (Perry et al. 1997, Stennicke and Salvesen 1997, Takahashi et al. 1996), or by changing cellular redox potential (Nakatani et al. 2000).

Another explanation could be that cell proliferation and apoptosis are tightly coupled processes (Evan and Littlewood 1998). Blockage of proliferation with serum deprivation leads to apoptosis rather than growth arrest in c-myc transfected fibroblasts (Evan et al. 1992). However, studies are needed to investigate whether such a mechanism exists in normal proliferating or differentiating cells.

**Collagen II mRNA expression**

A strong signal of collagen II mRNA was observed in the proliferative and prehypertrophic zones of the normal growth plate. In contrast, the signal in the hypertrophic zone was weaker on d 3 and totally diminished on d 7. The distribution pattern of collagen II mRNA on d 7 was similar to that previously described in 8 to 25 day-old chickens (Chen et al. 1993, Pines et al. 1998). Because expression of collagen II
mRNA is greatly reduced during chondrocyte hypertrophy, it has been used as a marker for non-hypertrophic cells (Chen et al. 1993, Pines et al. 1998). However, this study demonstrated that a considerable amount of collagen II mRNA was expressed by hypertrophic chondrocytes in the growth plate of chickens of 3 day post-hatch. A similar observation has been made in newly formed hypertrophic chondrocytes of the embryonic chick vertebral body (Iyama et al. 1994). Therefore, collagen II mRNA is not exclusively associated with non-hypertrophic chondrocytes, especially in the cartilage of embryonic or newly hatched chickens.

Zinc deficiency significantly weakened the signal of collagen II mRNA in the growth plate. On d 3, the reduction was universal to all of the chondrocytes in the proliferative and prehypertrophic zones. Thus, the decrease in collagen II mRNA may not be associated with premature cell differentiation, but could be due to a direct effect of zinc deficiency on the gene expression or mRNA stability. By d 7, the message was greatly decreased in the areas remote from blood vessels, which may be explained by the reduced cellularity and ongoing apoptosis. Zinc deficiency has been shown to reduce collagen synthesis in the epiphyseal cartilage (Suwarnasarn et al. 1982) and skin of rats (McClain et al. 1973), but the mechanism is unknown. This study suggested that zinc deficiency affected collagen metabolism through changing mRNA levels.

**Implications of the study**

1. The growth plate of chicken is a good model to study the effects of zinc deficiency on cell proliferation, differentiation and apoptosis. This study demonstrated
that zinc deficiency inhibited proliferation, promoted differentiation, and induced apoptosis of chondrocytes in the growth plate of young chickens. These effects occurred very rapidly (after newly hatched chickens consuming a low zinc diet for only 3 days). It is unclear whether single or multiple defects are responsible for zinc deficiency-induced cellular changes. Many in vitro and in vivo studies have been carried out to unveil the mechanisms by which zinc deficiency affects cell proliferation, differentiation or apoptosis (Chesters 1997, MacDonald 2000, Truong-Tran et al. 2000). However, most of systems have been used to demonstrate only one effect of zinc deficiency. The conclusions of the studies using such systems are far from complete, considering that inhibition of cell proliferation and induction of apoptosis could be one integrated process. The chicken growth plate is not only an in vivo system, but also shows rapid multiple responses. Therefore, the chicken growth plate is a good model to investigate whether single or multiple defects are involved in initiation of zinc deficiency-induced pathological changes.

Advances have been made in uncovering control of the cell cycle in growth plate chondrocytes (LuValle and Beier 2000). Future studies should address whether zinc deficiency affects the activities of the important molecules in the control process. The outcome will facilitate our understanding of zinc biology as well as the mechanisms for interaction of the nutrient and endochondral bone formation.

2. **Longitudinal bone growth may be very sensitive to zinc status.** The present study demonstrated that decreased supply of zinc first impaired cell proliferation remote from the blood vessels. The growth plate in animals is an avascular or poorly
vascularized tissue. Therefore, this study suggests that longitudinal bone growth may be especially sensitive to zinc status. Human studies have shown that a zinc supplement increases the growth rate of apparently normal infants and young children (Hambidge 2000), indicating that there exists a status of marginal zinc deficiency that only affects growth. These findings are consistent with the results of the present study.

3. Manipulation of zinc status may benefit cancer treatment. This study demonstrated that zinc deficiency inhibited cell proliferation and induced cell apoptosis in the growth plate tissue. In humans, solid tumors have some characteristics similar to the growth plate. Firstly, cells in both types of tissues rapidly proliferate. Secondly, solid tumors are avascular up to 1 – 2 mm in diameter (Folkman 1971, Kerbel 2000). Because a short term of zinc deficiency affected cell proliferation and survival of growth plate chondrocytes, growth of solid tumors could also be substantially restricted under zinc deficient conditions. It has been reported that zinc deficiency significantly reduces hepatoma growth in rats (Baker and Duncan 1983). However, relative long term zinc deficiency has adverse effects such as compromising immune function (Fraker et al. 2000). It is possible that repeated short-terms of zinc deficiency may control tumor growth and at the same time avoid adverse effects of zinc deficiency.

Summary

The present study demonstrated that dietary zinc deficiency inhibited chondrocyte proliferation, promoted cell differentiation and induced apoptosis in the chicken growth plate within a few days. Changes in the zinc status primarily affected cells remote from
the blood supply. This effect may not only explain the growth plate lesion in avian species, but also could account for significant reduction of longitudinal bone growth in zinc deficient animals. Future studies are needed to further investigate the molecular mechanisms responsible for the effects of zinc deficiency on cell proliferation. Since zinc deficiency impairs the transition of proliferating cells from G1 phase to S phase (Chesters et al. 1989), it would be of interest to study whether zinc deficiency affects the levels of cyclin D and cyclin A, which are responsible for the transition. The outcome of these studies will facilitate our understanding of zinc biology and the influence of nutrients on longitudinal bone formation.
Chapter 3

INFLUENCE OF MATERNAL ZINC STATUS AND DIETARY ZINC TREATMENT ON THE LONGITUDINAL BONE GROWTH OF YOUNG GROWING RATS

ABSTRACT

The purpose of this study was to investigate the influence of maternal zinc deficiency during lactation and dietary zinc treatment after weaning on the longitudinal bone growth of young growing rats. Sprague-Dawley dams were fed either a zinc-adequate (34 ppm zinc) or a low zinc (4 ppm zinc) purified diet throughout lactation. After weaning at 22 days postpartum, pups received dietary treatment for another 16 days. Pups from zinc-sufficient dams were fed the zinc-adequate diet (Control), while pups from zinc-deficient dams were divided into 3 groups: one group received a low zinc diet (2 ppm zinc) and the other two groups were fed the zinc-adequate diet with (pair-fed) or without (ad libitum-fed) food restriction. Maternal zinc deficiency significantly affected the growth of suckling pups. At age of 21 days, the average body weight, femur length, and femur dry weight were 30.1 g, 16.0 mm, and 47.4 mg for the pups from zinc-deficient dams, respectively, and 56.5 g, 18.9 mm, and 76.3 mg for the pups from zinc-sufficient dams, respectively (p<0.05). Furthermore, dietary zinc deficiency after weaning completely prevented growth of pups from zinc-deficient dams. Growth of pair-fed animals was also severely impaired. In contrast, the growth rate of ad libitum-fed animals was similar to that of the control animals. At the end of the study, femur length, femur dry weight, and the width of tibial growth plate were significantly lower in zinc-
deficient and pair-fed rats as compared to ad libitum-fed and control animals. No histological differences were observed in the growth plate between zinc-deficient and pair-fed rats. In conclusion, maternal zinc deficiency during lactation and dietary zinc restriction after weaning severely impaired the longitudinal bone growth of young growing rats. However, this effect was largely duplicated with food restriction.
INTRODUCTION

It is well known that zinc deficiency depresses food intake and impairs the growth of humans and animals (Cousins 1996). One of the growth parameters, longitudinal bone growth is greatly reduced in a zinc deficient state (Prasad et al. 1963, Todd et al. 1934, O'Dell et al. 1958, Leek et al. 1988, Golub et al. 1996). The effect of zinc deficiency on skeletal development has been linked to decreased activities of growth plates. The width of the growth plate is narrowed in zinc deficient animals (Follis et al. 1941, O'Dell et al. 1958). However, this effect can be duplicated with pair-feeding. A number of studies have demonstrated specific effects of zinc deficiency, which cannot be explained by reduction in food intake. For example, it was reported that zinc deficiency disrupted the structure of the tibial growth plate and decreased its shearing strength in young rats (Suwarnasarn et al. 1982). Disorganized chondrocytes have also been shown in the growth plate of zinc deficient rat fetus (daCunha Ferreira et al. 1991) and chicks (Young et al. 1958, Westmoreland 1971). Growth plates with such severe lesions would provide a desirable model to further study the molecular and cellular changes caused by zinc deficiency. However, it has been very difficult to produce severe growth plate lesions in post-weaning rats (Whitelock 1998).

Whitelock (1998) further studied effects of maternal diets containing various amounts of zinc (<1, 2, 4, 6 and 30 ppm zinc) on the bone growth of suckling pups. Results showed that the growth rate of pups was directly correlated with maternal dietary zinc levels. When dams consumed a diet containing 6 ppm zinc, the growth of suckling
pups was slightly affected, namely, the body weight was reduced less than 10% compared to the control pups from dams receiving a diet containing 30 ppm zinc, while the tibial length and the width of the growth plate were not different between the two groups. When maternal dietary zinc level was lowered to 4 ppm, the body weight of pups was reduced by 40% and longitudinal bone growth was also significantly impaired. In addition, the tibial zinc content of the pups was reduced to the lowest level. Further lowering maternal dietary zinc levels caused more reduction in the growth of pups, but not the tibial zinc content. In a litter of pups nursed by dams receiving a very low zinc diet (<1 ppm zinc), the integrity of the growth plate was severely affected. However, duplication of this effect was unsuccessful due to cannibalistic behavior of dams fed the very low zinc diet.

We hypothesized that severe zinc deficiency could lead to formation of lesions in the growth plate. To create such a zinc status in rats, we designed an experiment, in which zinc deficiency was extended from suckling to weanling period. During the suckling period, dams were fed a diet containing 4 ppm zinc to produce a moderate zinc deficient status in pups, yet avoid cannibalism. After weaning, pups were fed a low zinc diet containing 2 ppm zinc. Changes in the longitudinal bone growth and growth plate integrity were examined.
MATERIALS AND METHODS

Animals care

Sprague-Dawley female and male rats weighing 200-250 were obtained from a commercial supplier (Hilltop Lab Animals, Scotsdale, PA). Females were caged together with males for four days and then housed individually in suspended, stainless-steel cage. Deionized, distilled water was provided in acid washed glass bottles, plugged with silicon stoppers fitted with stainless-steel sipper tubes. Diets were provided in acid-washed, glass feed jars with stainless-steel collars. The room was maintained at 20-23°C with a 12-hour light/dark cycle. All animal care and surgical procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University (IACUC #98R077-0).

Experimental design

This experiment was designed to assess the effect of dietary zinc deficiency during suckling period and subsequent weanling period on the bone growth of rat pups. The feeding scheme is shown in (Figure 23). Dams were fed a pelleted, non-purified commercial diet ad libitum through gestation and then switched to purified egg white based diets after parturition. Five lactating dams were fed a low zinc diet containing 4 mg zinc per kg diet (4 ppm, by analysis) and three others were fed a zinc-adequate diet containing 34 ppm zinc ad libitum. Within two days of parturition, the litter size was adjusted to 8 pups per litter. At 22 days of age, the rat pups were weaned and housed
individually in suspended, stainless-steel cage.

Eight weanlings from the control dams (CTR) were fed the zinc-adequate diet and allowed free access to food. Twenty-four weanlings from the zinc deficient dams were randomly divided into three groups. Rats in one group (Zn-) received a diet containing 2 ppm zinc and animals in the other two groups were fed the zinc-adequate diet with (Pairfed, PFZn+) or without (ad libitum-fed, ALZn+) food restriction. PFZn+ rats were individually paired with rats in the Zn- group and fed the amount consumed in the previous 24 hours by their zinc deficient counterpart. Deionized, distilled water was
provided ad libitum. Feed intakes were recorded daily and rats were weighed every other day.

**Experimental diets**

The composition of purified diets is given in Table 2. Dietary zinc requirements for lactating rats and weanlings are 25 and 12 mg zinc per kg diet, respectively, when egg white is the main source of protein (Subcommittee on Laboratory Animal Nutrition et al. 1995, Fosmire et al. 1977, Rogers et al. 1985, Wallwork et al. 1981, Williams and Millis 1970). Studies of Whitelock (1998) have shown that the growth of suckling pups was severely compromised when lactating rats consumed a diet containing 4 ppm or less zinc. Zinc levels used in this study were chosen according to the above data. The vitamin mix met the AIN-76 recommendations. However, additional biotin was added to compensate for the reduced absorption of biotin by undenatured avidin in the egg white. The mineral mix complied with the AIN-76 recommendations (American Institute of Nutrition 1977) except that the amount of zinc in the diet was used as a variable (shown in Table 3). Zinc carbonate (Baker Analyzed, J. T. Baker, Philipsburg, NJ) was used for zinc supplement. Dietary zinc concentrations were determined by flame atomic absorption spectrophotometry after samples were digested with concentrated nitric acid (Instra-analyzed, J. T. Baker) and heat (Clegg et al. 1981). The measured values of zinc concentration in the diets are shown in Table 4.
Table 2: Composition of diets fed to rats

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white solids¹</td>
<td>200</td>
</tr>
<tr>
<td>Corn starch³</td>
<td>325.884</td>
</tr>
<tr>
<td>Dextrose¹</td>
<td>162.942</td>
</tr>
<tr>
<td>Corn oil¹</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose¹</td>
<td>50</td>
</tr>
<tr>
<td>AIN-76 vitamin mix¹,²</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mix⁴</td>
<td>36.172</td>
</tr>
<tr>
<td>Choline bitartrate⁵</td>
<td>2</td>
</tr>
<tr>
<td>Biotin¹</td>
<td>0.0025</td>
</tr>
<tr>
<td>Variable Ingredients⁶</td>
<td>163</td>
</tr>
</tbody>
</table>

¹Harlan Teklad, Madison, WI.
²Composition of vitamin mix (g/kg mix): thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; niacin, 3.0; calcium pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; vitamin B12 (0.1% trituration in mannitol), 1.0; dry vitamin A palmitate (500,000 U/g), 0.8; dry vitamin E acetate (500 U/g), 10.0; vitamin D3, trituration (400,000 U/g), 0.25; menadione sodium bisulfite complex, 0.15; sucrose (fine powder), 981.08.
³Embassy Foods Service Corp., Pittsburgh, PA.
⁴Supplies (g/kg diet): calcium phosphate, dibasic, 22.859; sodium chloride, 2.59; potassium citrate, monohydrate, 7.70; potassium sulfate, 1.82; magnesium oxide, 0.84; manganese carbonate, 0.1225; ferric citrate, 0.21; cupric carbonate, 0.0105; potassium iodate, 0.0004; sodium selenite, 0.0003; chromium potassium sulfate, 0.0193.
⁵Sigma, St. Louis, MO.
⁶Includes zinc carbonate and sucrose. Amounts are shown in Table 3.
Table 3: Adjustments made in the diet composition to vary zinc levels

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Zinc supplemented (mg/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Zinc carbonate (g/kg diet)</td>
<td>0.0</td>
</tr>
<tr>
<td>Sucrose (g/kg diet)</td>
<td>163.000</td>
</tr>
<tr>
<td>Total (g/kg diet)</td>
<td>163.000</td>
</tr>
</tbody>
</table>

Table 4: Dietary zinc concentrations measured using flame atomic absorption spectrophotometry

<table>
<thead>
<tr>
<th>Zinc supplemented (mg/kg diet)</th>
<th>Zinc concentration measured (mg/kg diet)$^{1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.05 ± 0.16</td>
</tr>
<tr>
<td>2</td>
<td>4.04 ± 0.05</td>
</tr>
<tr>
<td>30</td>
<td>34.41 ± 0.74</td>
</tr>
</tbody>
</table>

$^{1}$The values represent means ± SD of duplicate measurements.
Tissue preparation and analysis

The weanling rats were killed after 16 days of feeding. Animals were anesthetized and killed with overdose of carbon dioxide. Femurs and proximal tibiotarsi were dissected. Femurs were dried at 70°C overnight and weighed. Tibiotarsi were fixed in 4% paraformaldehyde and embedded in paraffin. Longitudinal sections (5 µm thick) were cut on a microtome and stained with hematoxin-eosin. Growth plates were examined and photographed under a Nikon TMS microscope.

Statistical analyses

The experimental design was a completely random design during the suckling period with two levels of dietary zinc. During the weanling period, an incomplete block design was used. Weanling rats from zinc-deficient dams received three levels of dietary zinc, whereas the animals from control dams were only treated with one level of dietary zinc. Analysis of variance was conducted using general linear model program of Minitab Statistical Software (Minitab Inc., State College, PA). Tukey’s test was used to separate treatment means. Differences were considered to be significant at p<0.05.
RESULTS

Suckling rats and dams

The body weight and bone growth parameters of suckling pups are shown in Figure 24 and Table 5. Maternal dietary treatment significantly affected the growth of suckling rats. The body weight of zinc deficient pups was significantly lower than that of the controls after dams received dietary treatment for 9 days (Figure 24). At age of 21 days, the body weight, femur length and femur dry weight of zinc deficient pups were reduced 46.7%, 15.3% and 37.9%, respectively, as compared to the parameters of control animals. Except the reduction in growth, no other signs of zinc deficiency were observed in zinc deficient pups.

Dams receiving the low zinc diet (Zn-) consumed less feed than dams receiving the zinc-adequate diet (CTR) (Figure 25). As a result, the body weight of Zn- dams gradually declined during the lactating period, while the body weight of CTR dams remained relatively constant (Figure 26). All five Zn- dams lost hair and had a poor hair coat, but only one developed severe parakeratosis on paws and legs.

Weanling rats

Feed intake of weanling rats is shown in Figure 27. When fed the zinc-adequate diet ad libitum, weanling rats derived from both zinc-deficient litters (ALZn+) and zinc-adequate litters (CTR) gradually increased their feed intake over the experiment period,
Figure 24: Influence of maternal dietary treatment on body weight of suckling rat pups.

Dams consumed a pelleted commercial diet during pregnancy (Figure 23). After giving birth, three dams were switched to a zinc-adequate purified diet containing 34 ppm zinc (control litters, CTR) and five others were fed a low zinc diet containing 4 ppm zinc (zinc-deficient litters, Zn-). Each litter had 7 or 8 pups. Values represent means ± SD.

*The mean of CTR litters is significantly higher than that of Zn- litters (p<0.05).
**Table 5**: Effects of maternal zinc status on body weight and bone growth parameters of suckling rat pups at age of 21 days

<table>
<thead>
<tr>
<th>Maternal dietary group</th>
<th>Suckling pups</th>
<th>Body Weight (g)</th>
<th>Femur length (mm)</th>
<th>Femur dry weight (mg)</th>
<th>Tibia growth plate width (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>56.5 ± 1.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.9 ± 0.311&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.3 ± 5.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>700 ± 100</td>
</tr>
<tr>
<td>Zn-</td>
<td></td>
<td>30.1 ± 1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.0 ± 0.134&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.4 ± 1.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>425 ± 25</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values represent means ± SEM. Values with different superscript letters in the same column are significantly different from each other at P < 0.05. For body weight, Control n=3 and Zn- n=5 (litters with 7 or 8 pups in each litter). For femur length and dry weight of each group, n=3 (litters with 2 pups examined in each litter). For tibia growth plate width of each group, n=2.

<sup>2</sup>Control pups derived from dams fed a zinc-adequate purified diet containing 34 ppm zinc and Zn deficient pups (Zn-) from dams fed a low zinc diet containing 4 ppm zinc during the suckling period.
Figure 25: Effects of dietary treatment on daily feed intake of dams.

Dams consumed a pelleted commercial diet during pregnancy. After giving birth, three dams (CTR) switched to a zinc-adequate purified diet containing 34 ppm zinc and five others (Zn-) were fed a low zinc diet containing 4 ppm zinc. Values represent means ± SEM.
Figure 26: Effects of dietary treatment on body weight of dams.

Dams consumed a pelleted commercial diet during pregnancy. After giving birth, three dams (CTR) switched to a zinc-adequate purified diet containing 34 ppm zinc and five others (Zn-) were fed a low zinc diet containing 4 ppm zinc. Values represent means ± SEM.
Rat pups derived from control and zinc deficient litters, dams of which were fed a zinc-adequate and a low zinc diet during suckling period, respectively (Figure 23). Pups were weaned at 22 days of age (day 0) and individually housed. Those from control litters (CTR) were fed a zinc-adequate diet containing 34 ppm zinc. Those from zinc deficient litters were divided into three groups: one of which fed a low zinc diet containing 2 ppm zinc (Zn-), the other two fed the zinc adequate diet with (pairfed, PFZn+) or without (ad libitum, ALZn+) food restriction. Values represent means ± SD (n=8). PFZn+ group (not shown) was individually paired with Zn- group and fed the amount consumed by Zn- rats during the previous day.

Figure 27: Influence of maternal zinc status and subsequent dietary treatments on feed intake of weanling rats.
whereas an opposite trend was shown in the animals receiving the low zinc diet (Zn-).

Zinc deficiency completely prevented Zn- weanlings from growing (Figure 28). However, the body weight of pair-fed animals (PFZn+) gradually increased in the first 8 days of feeding and then plateaued. Because of the body weight gain, PFZn+ rats were significantly heavier than Zn- rats after d 6 (p<0.05). ALZn+ and CTR rats grew at a similar growth rate, but the initial differences in the body weight between the two groups remained throughout the study.

All Zn- weanlings experienced hair loss and developed skin lesions on the abdomen (Figure 29). The femur length, femur dry weight, and tibial growth plate width were all significantly lower in Zn- and PFZn+ rats as compared to ALZn+ and CTR rats (Table 6). In H & E staining of longitudinal sections of tibial growth plates, no obvious histological differences were observed between Zn- and PFZn+ animals (Figure 30).

**DISCUSSION**

**Differential effects of zinc deficiency on rat and chicken growth plates**

Maternal zinc deficiency during lactation and subsequent dietary zinc restriction after weaning significantly reduced the growth plate width and impaired the longitudinal bone growth of young growing rats. However, these effects were largely duplicated with feed restriction. Furthermore, no differences were observed in the growth plate structure between zinc-deficient and pair-fed rats. Therefore, the apparent effects of zinc
Figure 28: Influence of maternal zinc status and subsequent dietary treatment on body weight of weanling rats.

Rat pups derived from control and zinc deficient litters, dams of which were fed a zinc-adequate and a low zinc diet during suckling period, respectively (Figure 23). Pups were weaned at 22 days of age (day 0) and individually housed. Those from control litters (CTR) were fed a zinc-adequate diet containing 34 ppm zinc. Those from zinc deficient litters were divided into three groups: one of which fed a low zinc diet containing 2 ppm zinc (Zn-), the other two fed the zinc adequate diet with (pairfed, PFZn+) or without (ad libitum, ALZn+) food restriction. Values represent means ± SD (n=8). Values with different letters on the same day are significantly different from each other at P < 0.05.
Figure 29: Zinc deficiency-induced skin lesions on weanling rats.

Rat pups derived zinc deficient litters, dams of which were fed a low zinc diet during suckling period (Figure 23). Pups were weaned at 22 days of age and individually housed. Zn- rats fed a low zinc diet containing 2 ppm zinc. PFZn+ rats were individually paired with Zn- rats and fed the amount consumed by Zn- rats during the previous day but a zinc-adequate diet containing 34 ppm zinc. Note the Zn- rat developed severe skin lesions after 16 days of dietary treatment.
Table 6: Body weight and bone growth parameters of weanling rats after 16 days of dietary treatment1

<table>
<thead>
<tr>
<th>Dietary treatment2</th>
<th>Body Weight (g)</th>
<th>Femur length (mm)</th>
<th>Femur dry weight (mg)</th>
<th>Tibia growth plate width (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>142.7 ± 3.35a</td>
<td>26.5 ± 0.311a</td>
<td>212.1 ± 7.22a</td>
<td>512.5 ± 12.5a</td>
</tr>
<tr>
<td>ALZn+</td>
<td>111.0 ± 3.28b</td>
<td>24.5 ± 0.828a</td>
<td>157.3 ± 12.6b</td>
<td>518.8 ± 12.0a</td>
</tr>
<tr>
<td>PFZn+</td>
<td>39.2 ± 1.20c</td>
<td>20.4 ± 0.265b</td>
<td>100.0 ± 3.25c</td>
<td>218.8 ± 12.0b</td>
</tr>
<tr>
<td>Zn-</td>
<td>30.1 ± 0.87d</td>
<td>18.3 ± 0.545b</td>
<td>71.9 ± 6.52c</td>
<td>231.3 ± 6.25b</td>
</tr>
</tbody>
</table>

1Values represent means ± SEM. Values with different superscript letters in the same column are significantly different from each other at P < 0.05. For body weight of each group, n=8; for other parameters, n=4.

2Rat pups derived from control and zinc deficient litters, dams of which were fed a zinc-adequate and a low zinc diet during suckling period, respectively (Figure 23). Pups were weaned at 22 days of age and individually housed. Those from control litters (CTR) were fed a zinc-adequate diet containing 34 ppm zinc. Those from zinc deficient litters were divided into three groups: one of which fed a low zinc diet containing 2 ppm zinc (Zn-), the other two fed the zinc adequate diet with (pairfed, PFZn+) or without (ad libitum, ALZn+) food restriction.
Figure 30: Longitudinal sections of tibial growth plates (H & E stained) from weanling rats after 16 days of dietary treatment.

A: CTR; B: ALZn+; C: PFZn+; D: Zn-. Bar = 100mM. Magnification 100X. (P: proliferative zone; PH: prehypertrophic zone; H: Hypertrophic zone.)

Rat pups derived from control and zinc deficient litters, dams of which were fed a zinc-adequate and a low zinc diet during suckling period, respectively (Figure 23). Pups were weaned at 22 days of age and individually housed. Those from control litters (CTR) were fed a zinc-adequate diet containing 34 ppm zinc. Those from zinc deficient litters were divided into three groups: one of which fed a low zinc diet containing 2 ppm zinc (Zn-), the other two fed the zinc adequate diet with (pairfed, PFZn+) or without (ad libitum, ALZn+) food restriction.
deficiency on skeletal development were similar to the effects of reduction in feed intake.

The results of this study were different from that of the study described in chapter 2. In the latter, severe growth plate lesions were induced in the chicken growth plate by zinc deficiency. These differential effects could be explained by the following differences between chickens and rats.

Firstly, the age of animals was different when they received severe zinc restriction. The rats were fed a low zinc diet after weaning, while the chickens received dietary treatment immediately post-hatch. Moderate zinc deficiency of rat pups during the suckling period did not induce growth plate lesions, which was consistent with the study of Whitelock (1998). On the other hand, weanlings could have passed the critical stage for bone development and the growth plate structure may not be susceptible to effects of zinc deficiency post-weaning (Whitelock 1998).

Secondly, the width of zinc deficient growth plates in rats was much narrower than that in chickens (Figure 31). It was demonstrated, in the study on chickens, that cellular activities were influenced by the distance between cells and the blood supply. In a zinc deficient state, growth plate chondrocytes remained relative normal near blood vessels within a distance of approximately 100µm, while cells further away became apoptotic. The growth plate width of zinc deficient chickens was about 500µm. Even though blood vessels penetrate the chicken growth plate, the distance between two adjacent vessels is usually more than 200µm. Therefore, there existed remote areas where
Figure 31: Schematic diagrams of longitudinal sections of the growth plate from zinc deficient chickens and rats.

In the diagram for chickens, Ar: articular cartilage; BV: blood vessel; Dv: distance between two adjacent vessels; H: Hypertrophic zone; L: lesion area; N: normal appearing area; Wc: the width of the chicken growth plate without hypertrophic zone. In the diagram for rats, 2OC: secondary ossification center; EB: epiphyseal bone trabeculae; EV: epiphyseal vessel; MV: metaphyseal vessel; Wr: the width of the rat growth plate.

Chicken: Normal appearing areas (N) are located adjacent to articular cartilage or near blood vessels within a distance of 100 mm. Lesion areas (L) are remote from articular cartilage and blood vessels, where cells are apoptotic and the cell density is greatly reduced. The width of the growth plate without hypertrophic zone of zinc deficient chickens (Wc) was about 500 mm on d 7 (Figure 5). The distance between two adjacent vessels (Dv) is usually 300 to 500 mm.

Rat: The growth plate width of zinc deficient rats (Wr) was about 230 mm at end of the study (Table 6).
chondrocytes were susceptible to the effect of zinc deficiency. In contrast, the growth plate width of zinc deficient rats was about 230µm at the end of the study (Table 6). Although the rat growth plate is an avascular tissue, the epiphyseal and metaphyseal vessels nourish the rat growth plate from proximal and distal sides, respectively (Kuettner and Pauli 1983). Thus, zinc supply in a deficient state could be still sufficient to support chondrocyte activities in the narrowed growth plate and, as a result, no lesion could be seen.

**Zinc deficiency versus pair-feeding**

Dietary zinc deficiency completely prevented pups of zinc deficient dams from growing during 16 days of post-weaning period. The growth of pair-fed animals was also severely impaired. Although the effects of zinc deficiency and pair-feeding on animal growth were similar, the underlying mechanisms could be different. Firstly, force-feeding a zinc deficient animal did not restore growth (Chesters and Quantermann 1970, Flanagan 1984). Secondly, oral administration of a synthetic progestin, megestrol acetate, to zinc deficient rats significantly increased food intake, but had no effect on the growth rate (Browning et al. 1998). Therefore, an inherent metabolic defect rather than reduction in feed intake may account for impaired growth due to zinc deficiency (Chesters 1997).

**Zinc repletion**

When pups from zinc deficient dams were fed the zinc adequate diet ad libitum after weaning, they grew at a growth rate similar to that of pups from zinc adequate dams.
However, the initial differences in the body weight at weaning remained between the two groups throughout the experiment. This is consistent with an observation of Mutch and Hurley (1974). Furthermore, zinc repletion to severe deficient rats did not only improve the growth, but also eliminated overt signs of zinc deficiency (Swenerton and Hurley 1968). However, zinc repletion did not recover the earlier reduction in growth caused by zinc restriction.

Conclusions

Maternal zinc deficiency during lactation and subsequent dietary zinc restriction after weaning significantly reduced the growth plate width and impaired the longitudinal bone growth of young growing rats. However, zinc deficiency did not induce histological changes in the rat growth plate as it did in the chicken growth plate. Differences in the age of animals exposed to severe zinc restriction and differences in the structure of growth plates between the two species may account for these variations in the outcome.
REFERENCES


VITA

Xibin Wang was born in Dingzhou, Hebei Province, People’s Republic of China on the 21st day of January 1965. He graduated from the College of Public Health, China Medical University and obtained a Bachelor’s degree in medicine in 1986. He continued his study in the Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine and graduated with a M. S. degree in nutrition in 1989. Afterwards, He worked as a research scientist in that institute for 5 years. In 1994, He joined the department of Nutrition, Food and Exercise Sciences in Florida State University to continue his graduate studies and obtained a M. S. degree in food and nutrition in 1996. He then transferred to the Intercollege Graduate Program in Nutrition at the Pennsylvania State University. He is expected to complete his Ph.D study in spring 2001. Xibin Wang was the recipient of student competitive research award in 1997 and Woot-Tsuen Wu Leung scholarship in 2000.