BIOMEDICAL AND ENVIRONMENTAL SCIENCES 15, 323-329 (2002)

Effect of Zinc on Bone Metabolism in Fetal Mouse Limb Culture[1]

LI YUN AND YU ZENG-LI

Department of Nutrition and Food Hygiene, Huaxi School of Public Health, Sichuan University, Chengdu 610041, China

Objective To determine the effects of zinc-deficiency and zinc-excess on bone metabolism. **Methods** We developed the culture model of fetal mouse limbs (16th day) cultivated in self-made rotator with continuing flow of mixed gas for six days *in vitro*. The cultured limbs were examined by the techniques of ^{45}Ca tracer and X-roentgenography. **Results** The right limbs cultivated had longer bone length, higher bone density than the left limbs uncultivated from the same embryo; and histologically, the right limbs had active bone cell differentiation, proliferation, increased bone trabecula, clearly calcified cartilage matrix, and osteogenic tissue. Compared with the control group, the zinc-deficient group and zinc-excess (Zn^{2+}120 μmol/L) group contained less osteocalcin (BGP) and ^{45}Ca content, and lower AKP activity; whereas zinc-normal (Zn^{2+}45 μmol/L and Zn^{2+}70 μmol/L) groups contained more BGP and ^{45}Ca contents, and higher AKP (alkaline phosphatase) activity. **Conclusion** Both zinc-deficiency and zinc-excess can alter bone growth and normal metabolism. The results indicate that the culture model of fetal mouse limbs (16th day) *in vitro* can be used as a research model of bone growth and development.

Key words: Zinc excess; Zinc deficiency; Bone metabolism; Organic culture

INTRODUCTION

It is now more than 70 years since Fell's (Fell & Robinson, 1929) pioneer work on the organ culture of limb rudiments in which bone formation was studied for the first time *in vitro* systems[1, 2]. In spite of the evidence for growth of these bones, there seemed to be no new enchondral or periosteal bone formation. Nor was there evidence of bone marrow development. Johnson[3] demonstrated that explants mouse limb buds aged 15 days developed a marrow cavity and showed signs of endochondral ossification for 9 days *in vitro*. *In vitro* systems are routinely used for the study of bone physiology and the effects of hormones on bone. But it has not been reported whether bone density can be measured *in vitro* in the cultured limbs.

Zinc plays an important role in maintaining bone metabolism and homeostasis. Because people in China and other developing countries mainly depend on cereal as staple food, which contains low-zinc and many factors interfering with zinc absorption, there is a high occurrence of zinc deficiency, and supplement of zinc is becoming indispensable, especially

[1] This work was supported by Grant 39600122 from the National Natural Science Foundation of China.

Biographical note of the first author: Li Yun, Ph.D, has been researching nutrition toxicology, especially teratology and teratogenicity of trace elements. Li Yun has been endowed by National Natural Science Foundation of China, and now mainly studies the effects of zinc deficiency or excess on bone development by means of *in vitro* culture method.

0895-3988/2002
CN 11-2816

in women and children. However, if supplement of zinc is improperly done, zinc-excess will result. Epidemiological data[4] and laboratory studies[5-7] showed that zinc-deficiency could affect animal and human growth, delay bone age and result in bone malformation. Also zinc-excess can lead to bone dysplasia in rats[6,7]. However, the effect of zinc-excess on bone metabolism, especially on bone density has not been determined. To determine how Zn affects bone metabolism, and whether bone density could be measured *in vitro* in the cultured limbs, a quick, simple, and reliable model was developed to culture fetal mouse limbs (16th day) *in vitro* and re-investigate the development of ossification.

MATERIALS AND METHODS

Reagent

Bigger's medium (BGJ-b), Hank's solution, TPEN (Zn-chelating agent: N' N' N' N-Tetrakis-(2-pyridymethyl) ethylenediamine), FBS (fetal bovine serum), $ZnSO_4$ and ^{45}Ca from Sigma, total albumen test box from Changzheng Scientific Corp. of Shanghai, BGP test box from Biologic Graduate School of North China.

Experimental Animals

Kunming strain female mice, weighing 25-35 g, were obtained from the West China University of Medical Sciences Breeding Laboratory. They had free access to food and water throughout the study.

Methods

Female mice were caged with fertile males at a ratio of 2:1, three in a cage, at 10:00 PM in an animal facility controlled for temperature, humidity, and lighting (12 h light:12 h dark). All females were examined next morning at 8:00 AM, those with observable vaginal sperm plugs were isolated and the day of finding the plug was noted as day 0 (E0) of pregnancy.

Pregnant female mice were killed by cervical dislocation on day 16 (E16) of development and embryos were removed in sterile condition. The forelimbs and hindlimbs were cut off at a point immediately adjacent to the body flank, just lateral to the somites. Forelimbs or hindlimbs from the same batch of litter embryos were pooled and transferred to 50mL bottles with 6 mL of culture medium in each bottle. Three limbs were randomly put in each bottle. Bottles were attached to a rotator at 30 rpm and 37℃ for 6 days with continuing flow of mixed gas. The culture medium was changed at 3 d intervals and the cultures were harvested after 6 d of incubation[7].

The culture medium used for the experiment comprised 90% by volume of commercially available Bigger's medium (BGJ-b) and 10% by volume of FBS (containing 150 μg/mL ascorbic acid, 100 U/mL penicillin, 100 μg/mL streptomycin, and 7.5 mmol/L Hepes-buffer pH 7.5). The mixed gas was composed of 50% O_2, 45%N_2, and 5% CO_2.

After 6 days of cultivation, limbs were removed and placed in Bouin's fixative overnight. Fixed limbs were decolorized (70% ethanol containing 50 drops/L NH_3H_2O) and rinsed with 70% ethanol. Fixation procedure was kept constant to minimize the histological variables in limb size and form induced by techniques.

Treatment

Establishing the culture model of mouse embryo limb. The experiment was divided

into two groups: the left limbs, which were decided as a control group, were not cultivated; the right limbs, which were decided as an experiment group, were cultivated for six days.

Study on the effect of Zn on bone metabolism. The experiment was divided into five groups: The control group (ZC) was in basic culture medium (20 μmol Zn^{2+}/L). The Zn-deficiency group (ZD) was in the basic culture medium with Zn-chelating agent (TPEN) of 20 μmol/L[18]. The supplemented-zinc groups Ⅰ (ZSⅠ), Ⅱ(ZSⅡ), and Ⅲ(ZSⅢ) were in the basic culture medium respectively with $ZnSO_4$ of 45 μmol/L, 70 μmol/L, and 120 μmol/L. For each group, 0.01mCi ^{45}Ca was added into the basic culture medium.

The cultivated limbs were homogenated with homogenizer. Then, total albumen was measured by the method of biuret reaction, and ^{45}Ca counted with FJ-2107P liquid scintillation counter, the content of osteocalcin measured by the method of radioimmunochemistry test, and the activity of alkaline phosphatase measured by the method of 4-NPP enzyme dynamics.

Bone density measure. Bone density of the fixed limbs was measured by taking photographs of X-ray (Senographe DMR at 22 KV, 48 mAs).

Histological analysis. Fixed limbs were wrapped by paraffine, sliced up and dyed by HE.

Methods of Analyzing

20 limbs were randomly selected from each group for analysis. We summarized all data as $\bar{x} \pm s$. A one-way analysis of variance and Newman-Keols multiple comparison of treatment groups were used to evaluate the data for statistical significance.

RESULTS

Establishing the Culture of Mouse Limb

After 6 days of cultivation, the bone (long bone) length and density of right limb were greater than those of the left limb (control group) (Fig.1), and compared with the control group, the right limb showed active bone-cell differentiation, proliferation, increased bone trabecula, more clearly calcified cartilage matrix and osteogenic zone (Figs. 2 and 3).

TABLE 1

The Diaphyseal Length of Fetal Mouse Long Bone, a Comparison Between Measurement of the Long Bone (ulna) Before Culture and After Being Cultured (mm) $\bar{x} \pm s$

	n	0 Day	6 Days
Length	10	0.95±0.12	1.88±0.16*

Note. *$P<0.01$ vs 0 day.

The Effect of Zn on Bone Metabolism and Development

Compared with ZC, ZSⅢ(120 μmol Zn^{2+}/L) and ZD showed decreased long bone density, whereas, ZS Ⅰ (45 μmol Zn^{2+}/L)and ZS Ⅱ (70 μmol Zn^{2+}/L) showed increased long bone density (Fig. 4).

A B

FIG. 1. A: Control limb (16-day-old left limb uncultivated); B: 16-day-old right limb cultivated for 6 d. The bone (long bone) length and density of right limb were longer and higher than these of the left limb.

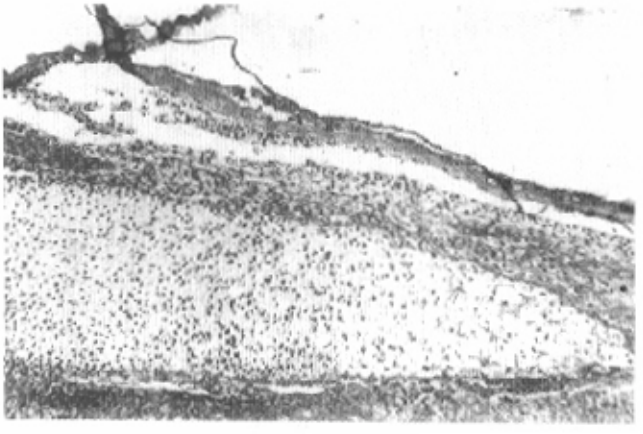

FIG. 2. Longtitudinal section through the radius of a 16-day-old left forelimb uncultivated. Note the vague osteogenic zone. (HE×60)

Compared with ZC, ZD showed cytochromation-contracted clot, cytomembrane rupture and matrix loss; ZS I and ZS II indicated that, in the zone of cartilage hyperplasia, the homogeneous cell cluster was abundant and distributed evenly, with cartilage matrix showing stronger basophilic stain reaction; ZSIII showed cell tumefaction and cytomembrane rupture.

The content of osteocalcin, ^{45}Ca, and the activity of alkaline phosphatase within the bone tissue were significantly higher in ZS I and ZS II, and significantly less in ZD and ZS III than in ZC (Table 2).

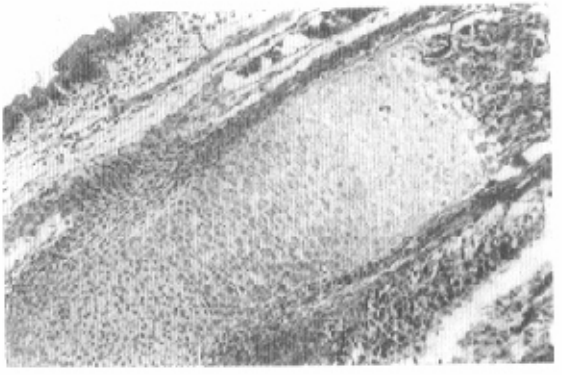

FIG.3. Longitudinal section through the radius of a 16-day-old right forelimb grown in culture for 6 d. Note the developing marrow cavity, increased bone trabeculae, more calcified cartilage matrix and osteogenic zone. (H&E × 66)

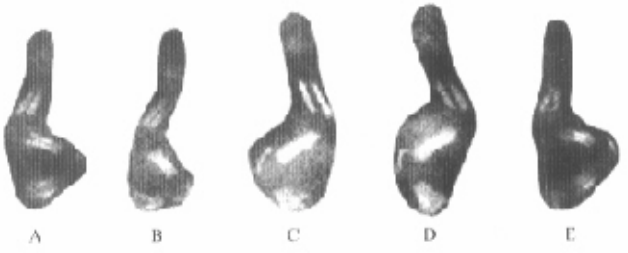

FIG.4. 16-day-old forelimbs cultivated for 6 days. A. Zn-deficiency group, B. Control group, C. Zn-supplemented group I , D. Zn-supplemented group II , E. Zn-supplemented groupIII.

TABLE 2

The Effects of Zn on the Content of BGP, AKP, and ^{45}Ca in the Bone Tissue

Group	Number	BGP /mg Protein	AKP μg/g Protein	^{45}Ca CPM/mg Protein
ZD	20	0.63 ± 0.02^{a}	151.20 ± 4.41^{a}	2665.92 ± 395.94^{a}
ZC	20	0.75 ± 0.01	183.35 ± 4.19	4318.47 ± 410.01
ZS I	20	1.02 ± 0.04^{a}	213.60 ± 3.6^{a}	5435.59 ± 533.72^{a}
ZS II	20	1.01 ± 0.03^{a}	210.00 ± 5.0^{a}	$5341.48 \pm 488.83a$
ZSIII	20	0.49 ± 0.02^{a}	155.10 ± 4.8^{a}	3548.88 ± 305.62^{a}

Note. ^{a} $P<0.05$ vs ZC (control group).

DISCUSSION

The results of this study indicate that using the culture system described, there is a measurable increase in diaphyseal length and visible increase in bone density of mouse

limbs after 6 d of cultivation. The 16-day-old explants showed histological signs of new bone formation, marrow cavity development, and calcification after 6 d in culture as described by Schwartz[9] and Johnson[3]. At this stage (16-day-old fetuses), calcification of the collagen matrix is just beginning. Thus, for the development of a quantitative assay to measure bone formation and resorption (observing the ^{45}Ca released from prelabelled bones) during bone development, the 16-day-old fetal mice bones would be the preferred samples. These bones grow well, and are mineralized enough prior to culture to incorporate injected ^{45}Ca which will then be released into the organ culture medium, serving as a good index for bone resorption[10]. An interesting observation was the change in long bone density (e.g. humerus, radius, and ulna) after 6 days' incubation (Figs.1 and 2).

The experiment reported above shows that the zinc-deficient and zinc-excess (Zn^{2+}120 μmol/L) can decrease the content of osteocalcin and ^{45}Ca, and bone density and the level of AKP activity in a rudiment removed for culture; whereas zinc-normal (Zn^{2+}45 μmol/L and Zn^{2+}70 μmol/L) can increase the contents of BGP and ^{45}Ca, and bone density and the level of AKP activity. It indicates that zinc-deficient and zinc-excess can deteriorate bone formation and ossification and only adequate zinc can benefit bone formation.

AKP is the marker enzyme of osteoblast, which can decompose phosphate compound to produce free phosphate, which can combine with calcium among the intercellular substance to form calcium orthophosphate taking part in regulating the process of calcification. AKP is a kind of zinc dependent enzyme. Zinc stabilizes its conformation and maintains its activity. When zinc is deficient, AKP will lose its conformation and function. It is through one of physiological mechanisms that zinc regulates bone metabolism through activating and synthesizing some zinc-metalloenzymes.

Leek *et al.*[11] reported that when Rhesus monkey was fed with food short of zinc during the gestational period and childhood, compared with the young who were fed with normal food, its bone showed hypocalcification, its epiphysis center appeared slow and X-ray demonstrated thinner bone matrix, decreased bone density, thinner and wider growth plate, and the bone features was similar to the hypocalcification resulted from the deficiency of $VitD_3$. These results are similar with our findings from cultured mouse embryo limb *in vitro*.

Bone formation is a complicated process, during which balance among trace elements is an essential condition for the normal bone development. Mg^{2+} is also an auxiliary factor for alkaline phosphatase. When Zn is excessively taken, it restrains bone tissue from absorbing Mg^{2+}, which also cuts down the activity of AKP[12]. The current study confirms that overdose of Zn-supplement can produce toxic effect on bone metabolism. It is the interaction of Zn and other trace elements together to hold the balanced condition that regulates bone metabolism. Excessively supplied zinc can deteriorate the bone loss induced from the diet of low copper diet, while properly supplied zinc can decrease the toxic effect of Cd, V, Ge, Se and Al on bone. It indicates the significance of keeping the homeostasis. So, it is necessary to make further study on the toxic dose of Zn when the salubrity of zinc is stressed.

REFERENCES

1. Fell, H.B. and Robinson, R. (1929). The growth, development and phosphatase activity of embryonic avian femora and limb buds cultivated *in vitro*. *Biochem. J.* **23**, 767-784.
2. Fell, H.B. (1964). The role of organ cultures in the study of vitamins and hormons. *Vitams Horm.* **22**, 81-98.
3. Johnson, D.R. (1980). Formation of marrow cavity and ossification in mouse limb buds grown *in vitro*. *J. Embryol. Exp. Morph.* **56**, 301-307.
4. Beattie, J.H. and Avenel, A. (1992). Trace element nutrition and bone metabolism. *Nutri Res Rev.* **5**, 167-188.
5. Rossi, L., Migliacco, S., Corsi, A., Marzia, M., Bianco, P., Teti, A., Gambelli, L., Cianfarani, S., Paoletti, F., and Branca, F. (2001). Reduced growth and skeletal changes in zinc-deficient growth rats are due to impaired

growth plate activity and inanition. *J. Nutr.* **131**(4), 1142-1146.
6. Li, Yun and Wang, Rui-Shu (1996). Studies of Teratogenicity on zinc deficiency and excess in pregnant rats. *Modern Preventive Medicine* **22**(3), 144-146.
7. Li, Yun and Wang, Rui-Shu (1997). Application of the mouse limb bud culture to study the teratogenicity by zinc deficiency or excess. *Acta. Nutr. Sinica* **19**(2), 196-201.
8. Zalewski, P.D., Forbes, I.J., and Giannakis, C. (1991). Physiological role for zinc in prevention of apoptosis. *Biochem. Int.* **24**, 1093-1101.
9. Schwartz, Z., Ornoy, A., and Soskolne, W.A. (1985). An *in vitro* assay of bone development using fetal long bones of mice: morphological studies. *Acta. Anat.* **124**, 197-205.
10. Schwartz, Z., Ornoy, A., and Soskolne, W.A. (1983). An *in vitro* model of bone remodelling. *Calcif. Tissue Int.* **Suppl.99**.
11. Leek, J.C., Keen, C.L., Vogler, J.B., Golub, M.S., Hurley, L.S., Hendrickx, A.G., and Gershwin, M.E. (1988). Long-term marginal zinc deprivation in rhesus monkeys IV. effects on skeletal growth and mineralination. *Am. J. Clin. Nutr.* **47**, 889-895.
12. Ciancaglini, P., Pizauro, J.M., Curti, C., Tedesco, A.C., and Leone, F.A. (1990). Effect of membrance moiety and magnesium ions on the inhibition of matrix-induced alkaline phosphatase by zinc ions. *Int. J. Bioche.* **22**, 747-751.

(*Received December* 20, 2001 *Accepted May* 25, 2002)