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ZINC AND ESSENTIAL FATTY ACIDS MODULATE BONE GROWTH AND METABOLISM IN RATS

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ABSTRACT: The effects of zinc and essential fatty acids (EFAs) on bone growth and metabolism were studied in young growing rats. Female weanling rats were maintained on diets deficient in EFAs, low in zinc (6 ppm) or both deficient in EFAs and low in zinc. Femur weights of rats maintained on EFA-deficient diet low-zinc diet or double-deficient diet were decreased by a factor of 0.32, 0.39 and 0.47 respectively, when compared with rats on control diet. The factor for the corresponding decrease in bone length was 0.08, 0.08 and 0.46 respectively.

Feeding of deficient diets to rats resulted in lower alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities. This reduction is due to reduced enzyme synthesis as well as loss of enzyme due to tissue decalcification. The low-zinc status aggravated EFA-deficiency, causing a higher reduction in bone growth as well as ALP and LDH activities. It is considered that: (a) the deficiencies of EFAs and zinc cause bone decalcification, and (b) ALP and LDH play significant roles in the early stages of the calcification process but that their activities are inhibited by EFA and zinc deficiency states.

Key Words: Essential fatty acids; Zinc deficiency; Bone growth; Alkaline phosphatase, Lactate dehydrogenase.

INTRODUCTION

The presence of a large amount of zinc in bone (1) raises the possibility that it plays an important role in the development of skeletal tissues. Previous studies (2,3) have shown that zinc deficiency in experimental animals causes considerable changes in bone growth and maturation. The stimulation and synthesis of some structural elements of bone tissue, namely collagen, chondroitin-4-sulphate and osteocalein are in some ways dependent on zinc (3-6). In zinc deficiency (7), there was a decrease in the activity of alkaline phosphatase, a zinc metalloenzyme in the osteoblast which functions to provide calcium deposition in bone diaphyses.

The work of Odutuga (2) indicated that both zinc and EFA deficiencies resulted in bone growth retardation. The mechanisms underlying the metabolic interaction between zinc and EFAs in animal tissues have been the focus of many investigations (8-12). In mammals, the conversion of linoleic acid to arachidonic acid and eventually prostaglandins has been postulated to require zinc (13,14). Some previous reports have more glaringly demonstrated that zinc deficiency in rat is able to interfere with intestinal absorption of fat (15,16) while some others have tried to explain the cause of imparied absorption of lipid and lipid soluble nutrients in zinc deficiency (17,18). Numerous studies on zinc deficiency in animal and human models showed prominent changes in tissue phospholipid content (2,19,20) and composition (21,22). Increasing susceptibility of membrane phospholipids to peroxidation in zinc deficiency would reduce the availability of EFAs not only for phospholipid synthesis but also for the synthesis of eicosanoids.

Since Odutuga (2) demonstrated the effect of zinc restriction and the deficiency of EFAs on bone tissue, there have been little or no focus on this area of research, and the exact mechanisms by which EFAs and zinc modulate metabolic activities in bone tissue remain unclear. This study was, therefore, conducted to further elucidate the effects of the deficiencies of zinc and EFAs on two enzymes in bone tissue, namely ALP which is required for bone calcification and LDH which is a marker enzyme in the cytosol and which is also of importance in energy metabolism.

MATERIALS AND METHODS

Animals and diets

A total of one hundred and twenty-four female white albino rats (*Rattus norvegicus*) weighing between 77.0 and 84.0g were used for the experiment. They were divided into four groups, each containing 31 animals and housed in plastic cages of stainless steel wire tops and bottoms. The animals were acclimatized for 24 hours before introducing the experimental diets. At the end of the fasting period, one animal was selected randomly from each group and sacrificed for analysis for day zero (the basal level). The remaining 30 rats in each group were maintained respectively on the following diets:
(a) EFA-deficient diet, (b) low-zinc (also referred to in the text as zinc deficient) diet, (c) low-zinc diet containing no EFAs and (d) control diet (containing adequate zinc and EFAs).

The composition of the diets is shown in Table 1. Groundnut cake was solubilized and treated with ethylenediamine-tetraacetic acid (EDTA) to remove zinc (23). The dried protein was then extracted with organic solvents as previously described to remove lipid (19). There was no lipid left in the protein. When added, zinc was supplied as $ZnCl_2$. The low-zinc diet and the diet adequate in zinc contained 6 ppm and 100 ppm zinc respectively (11). The fatty acid composition of the coconut oil used to compose the EFA-deficient diet has been previously reported (2). Diet adequate in EFAs contained soyabean oil. The soyabean oil used in this study contained palmitic acid (10.6%), stearic acid (4.0%), oleic acid (23.2%), linoleic acid (53.7%) and α-linolenic acid (7.6%). The diets and water were given *ad libitum*. There was no zinc in the water of the animals fed the low-zinc diet. All rats were fed their respective diets daily and sacrificed, five at a time, at week 1, 3, 5, 6, 7 and 8.

The femurs from each rat were removed, cleaned, weighed and the lengths were measured (2).

Protein determination

Protein concentration was determined by the biuret method (24).

Analysis of calcium and phosphorus

Known weights of pure bone samples, obtained as described above, were dissolved in 5N HCl (25) and aliquots analysed for calcium (26) and phosphorus (27) as well as the degree of mineralization (28).

Determination of enzyme activity

The excised femur was sawn into pieces, ground in sucrose buffer in a mortar and kept in the freezer overnight to autolyse (2). The supernatants obtained were used for enzyme assays. Enzymes were assayed spectrophotometrically. ALP (EC 3.1.3.1) activity was determined by the method of Wright *et al.* (30) and LDH (EC 1.1.1.27) by the method of Wroblewski and LaDue (31).

Statistical analysis

Analyses of variance were carried out to determine the statistical significance of the results.

Table 1: Composition of diets (in g/kg).

	+EFA+Zn	+EFA-Zn	-EFA+Zn	-EFA-Zn
Groundnut cake*	250	250	250	250
DL-Methionine	4	4	4	4
Corn starch	516	516	516	516
Cellulose	40	40	40	40
Sucrose	100	100	100	100
Soybean oil	40	40	-	-
Coconut oil (hydrogenated)	-	-	40	40
Mineral mix**	40	40	40	40
Vitamin mix ***	10	10	10	10

*Groundnut cake was defatted several times with petroleum ether and chloroform/ methanol (2:1 v/v) followed by EDTA treatment (19).

**The mineral mix contained (g/kg diet): NaCl (5.573); KH_2PO_4 (15.599); KI (0.032); $MnSO_4.2H_2O$ (0.178); $MgSO_4$ (2.292); $FeSO_4.7H_2O$ (1.078); $CuSO_4.5H_2O$ (0.019); $CoCl_2.6H_2O$ (0.001); $CaCO_3$ (15.258).

***The vitamin mix contained (g/kg diet): Thiamine (0.02); Riboflavin (0.022); L-ascorbate (1.00); Calcium panthothenate (0.066); Niacin (0.1); Pyridoxine-HCl (0.02); Folic acid (0.002); L-Inositol (0.11); p-Amino benzoic acid (0.11); Vitamin B_{12} (0.00003); Biotin (0.0004); Choline chloride (1.67); Vitamin A (0.011); Vitamin D (222 IU); Menadione (0.0001); α-Tocopherol (0.50)

RESULTS

The effect of the various deficient diets on bone growth and development in shown in Table 2. Compared with the controls, when rats were reared on a diet deficient in EFAs, low in zinc or both deficient in EFAs and low in zinc, the femur weight was decreased significantly by a factor of 0.32, 0.39 and 0.47 respectively ($P < 0.001$). The factor for the corresponding decrease in bone length was 0.08, 0.08 and 0.46 respectively ($P < 0.001$). It is apparent from these results that the bone weight was more decreased in zinc deficiency than in EFA deficiency. Both deficiencies, however, had similar effect on bone length gains. On the other hand, the double deficiency of zinc and EFAs had the most severe effect on bone weight as well as bone length gain.

The results of calcium and phosphorus analyses (Table 2) show that the animals fed the control diet, the EFA-deficient diet, the zinc-deficient diet and the diet deficient in both EFAs and zinc had molar ratios of calcium to phosphorus of 1.49 - 1.61. The molar ratio for EFA-deficient, zinc-deficient and double-deficient rat femur is 98.1, 96.3 and 95.0 per cent respectively of the control value. These results are statistically significant ($P < 0.001$).

The effect of consumption of the deficient diets on bone alkaline phosphatase activities is hown in Table 3. Compared with the controls, rats maintained on any of the deficient diets had considerable reductions in the activities of the enzyme beginning from the first week. Rats maintained on the double deficient diet had the most drastic reductions in ALP activity.

Table 2: Effect of zinc and EFA deficiencies on some parameters of bones of developing rats.

Diets	Weight of femur (mg)	Length of femur (cm)	Calcium (%)	Phosphorus (%)	Ca/P Molar Ratio
+EFA+Zn	278 ± 1.4	2.50 ± 0.05	35.56 ± 0.05	17.06 ± 0.21	1.61 ± 0.02
+EFA-Zn	169 ± 1.8	2.30 ± 0.03	32.52 ± 0.09	16.22 ± 0.37	1.55 ± 0.02
-EFA+Zn	189 ± 0.0	2.30 ± 0.03	34.00 ± 0.46	16.62 ± 0.18	1.58 ± 0.03
-EFA-Zn	148 ± 1.6	1.35 ± 0.02	31.72 ± 0.31	16.00 ± 0.12	1.53 ± 0.04

The results are the mean values for 10 analyses in each group (± SEM)

Table 3: Effects of diets deficient in EFAs and zinc on alkaline phosphatase activity of rat femur.

Weeks on diet	+EFA+Zn	+EFA-Zn	-EFA+Zn	-EFA-Zn
1	410.4 ± 10.2	409.3 ± 17.7	255.2 ± 14.8	189.7 ± 17.1
3	389.7 ± 5.4	227.6 ± 11.2	348.3 ± 8.1	293.1 ± 9.3
5	437.9 ± 8.5	269.0 ± 8.4	331.0 ± 9.7	151.7 ± 4.1
6	462.1 ± 9.3	293.1 ± 6.7	322.4 ± 2.1	77.6 ± 1.2
7	513.8 ± 11.0	158.6 ± 7.2	296.6 ± 6.5	69.0 ± 2.0
8	527.6 ± 10.1	162.1 ± 4.2	179.3 ± 4.4	70.0 ± 2.1

Enzyme activities are expressed as specific activities in nM/min/mg protein. Each value represents the mean of 10 determinations ± SEM. Specific activity of ALP at week 0 was 500.0 ± 6.4 nM/min/mg protein.

Table 4: Effects of diets deficient in EFAs and zinc on lactate dehydrogenase activity of rat femur.

Weeks on diet	+EFA+Zn	+EFA-Zn	-EFA+Zn	-EFA-Zn
1	52.0 ± 1.8	26.8 ± 2.1	34.0 ± 1.8	35.2 ± 3.0
3	51.2 ± 2.8	34.0 ± 2.2	36.4 ± 2.7	46.0 ± 3.3
5	49.2 ± 3.7	32.0 ± 1.7	39.6 ± 2.1	28.8 ± 1.3
6	42.0 ± 2.4	30.0 ± 1.8	37.2 ± 2.2	16.4 ± 0.8
7	38.4 ± 2.3	29.2 ± 2.1	34.4 ± 1.7	17.6 ± 0.6
8	37.2 ± 3.0	28.0 ± 2.4	32.4 ± 1.1	19.2 ± 1.5

Enzyme activities are expressed as specific activities in nM/min/mg protein. Each value represents the mean of 10 determinations ± SEM. Specific activity of LDH at week 0 was 12.0 ± 0.5 nM/min/mg protein.

At the end of the feeding period, the activity of the enzyme from rats fed zinc deficient, EFA-deficient and the double deficient diet was reduced to 30.7, 34.0 and 13.3 per cent, respectively, of the control value ($P < 0.001$). ALP activity at week 0 was 500.0 ± 6.4 nM/min/mg protein. It is important to note that at week 7 the ALP activity of the control started to stabilize and even exhibited a higher activity than that observed at week 0.

The levels of femur lactate dehydrogenase activities are as indicated in Table 4. Enzyme activity at week 0 was 12.0 ± 0.5 nM/min/mg protein. The enzyme activity rose conseiderably for all the dietary regimes right from the first week. Compared with the controls, rats reared on deficient diets had reduced lactate dehydrogenase activities. At the end of the feeding experiment, the activity of the enzyme from rats fed zinc deficient, EFA-deficient and the double deficient diet was reduced to 75.3, 81.7 and 51.6 per cent respectively of the control value ($P < 0.001$). These reductions, though considerable, are not as drastic as those observed for ALP under the same conditions.

DISCUSSION

It has been reported earlier (2) that when rats were made deficient in EFA, zinc or both, mean weights and lengths of bones decreased considerably. In the present study, it has been observed that the deleterious effect of deficient diets on the femur weight is in the order: double-deficient diet > zinc-deficient diet > EFA-deficient diet; and similarly on femur length: double-deficient diet > zinc-deficient diet = EFA-deficient diet. These findings would underscore the harmful effect of subsisting on a diet deficient in zinc, EFA or both.

The results of the present study indicate that feeding of diets deficient in either zinc, EFA or both to rats result in a significant reduction in alkaline phosphatase activity in the rat femur. Alkaline phosphatase is highly active in bone and it is involved in the cleavage of phosphate ions from organic ester linkage in the calcification process (32). The considerable reduction in the activity of this enzyme in young growing femur of rats in either zinc or EFA deficiency would, therefore, be expected to adversely affect the calcification process in the bone. Similarly, it has been reported (33) that rats fed a vitamin D_3-deficient diet had disturbed bone mineralization leading to rickets.

In our previous (2), it has been shown that when rats were fed zinc or EFA deficient diets for 8 weeks, bone mineral was still apatite in nature, but less calcium had been deposited. Results obtained from the present study indicate that the femur of rats maintained on the EFA-deficient, zinc-deficient or the double-deficient diet exhibited 98.1, 96.3 and 95.0 per cent degree of mineralization. This would indicate that some amount of calcium and phosphorus had been lost from the bone tissue. A calcium to phosphorus molar ratio of 1.5 - 1.63 is considered to occur in apatite, depending on how it is formed (2,34). It is suggested that the deficiency of either zinc/EFA may alter the availability and binding of calcium and phosphate ions at the calcification fronts. A long-term reduction in the activity of bone ALP may reduce the availability of phosphate ions necessary for calcium binding in hydroxyapatite formation. This may lead to disturbed bone calcification, especially in young growing individuals.

In both humans and animals, the deficiencies of zinc and EFA produce similar effects (2,11,19) owing to the essential role performed by zinc in EFA metabolism. Changes in lipid and fatty acid composition of bone and dental tissues have been reported in the deficiencies of both EFAs and zinc (2,19). These changes may affect the ability of the phospholipids (which have been implicated in the calcification process) to bind calcium (35,36), thereby giving rise to impaired or immature mineralization. It is suggested that it is this immaturity or hypomineralization that is partly responsible for the reduction in the weight and length of the bone tissue.

The effect of the deficiencies of zinc and EFA on LDH activity is similar to that observed for ALP. The reductions in the activity of this enzyme in the deficiency states were not as drastic as those observed for ALP. LDH is a cytosolic

enzyme. ALP and LDH are zinc metalloenzymes and zinc is required for their biologically active conformations. Restriction of zinc, therefore, would be expected to reduce the amount of the enzymes in the tissue. The reduction in the activities of these enzymes may in addition be attributable to loss of enzyme owing to bone decalcification. This is in agreement with the detection of ALP on decalcified sections of tibial bone collar fetuses (37). A reduction in the activity of LDH would probably indicate impairment of the transport and deposition of Ca^{2+} in the bone; an energy requiring process. ALP and LDH have been detected in the growth zone of young rat ribs (38) indicating that these two enzymes are most likely actively involved in the process of bone growth.

In EFA deficiency, there are no EFAs to convert to prostaglandins (PGs); whereas in zinc deficiency, the EFAs are accumulated and not metabolised to PGs. In either case, there is a considerable decrease in circulating PGs necessary for bone growth and development. It is inferred that deficiency of either zinc or EFA leads to deficiency of PGs which act on target bone cells as well as influence changes in membrane fluidity. Prostaglandins of the E series are powerful mediators of tooth and bone resorption and formation. It is inferred from the present work that their deficiency (owing to deficiency of linoleic acid) may initiate bone decalcification; and that both ALP and LDH may also be lost together with decalcified sections of bone tissue.

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