Inadequate Copper Intake Reduces Serum Insulin-Like Growth Factor-I and Bone Strength in Growing Rats Fed Graded Amounts of Copper and Zinc1–3

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ABSTRACT This study examined the effects of graded intakes of zinc (Zn) and copper (Cu) on serum insulin-like growth-factor-I (IGF-I) concentration and bone quality in growing rats. Using a 3 × 4 factorial design, weanling male Sprague-Dawley rats were randomly assigned to 12 groups (n = 7 per group) and were fed one of nine modified AIN-93G basal diets with varying amounts of Cu (0.3, 3 and 10 μg/g) and Zn (5, 15 and 45 μg/g) for 6 wk. A group of rats was pair-fed to each low Zn group. Although dietary Zn mainly influenced body weights (P < 0.0001), dietary Cu was the main determinant of most of the variables related to bone quality. Low Cu intake reduced serum IGF-I and femur breaking force and ultimate stress (by 27, 14 and 7%, respectively; P < 0.05) and increased bone IGF-I concentration (by 62%; P = 0.0001). Low Cu intake also increased femur nitrogen, hydroxyproline, hexosamine and calcium (Ca) concentrations of long bones (P < 0.05). Lumbar vertebrae dry weight and density were the lowest in the rats fed the low Cu diets (P < 0.001) and were higher in the rats fed high amounts of both Cu and Zn (P < 0.01). In summary, growing rats fed low and marginal Cu had lower serum IGF-I than those fed high dietary Cu. Bone strength was also reduced with low dietary Cu, despite compensatory changes in the bone matrix. In the presence of graded intakes of Cu, the effects of low dietary Zn were more pronounced on the spinal bones than the long bones.


KEY WORDS: • copper • zinc • bone strength • IGF-I • osteoporosis

A decreased serum concentration of insulin-like growth factor-I (IGF-I)5 is strongly associated with an increase in risk of osteoporotic fractures (1). This growth factor is essential for longitudinal bone growth because it stimulates the proliferation and differentiation of bone cells (2). Although growth hormone is the principal hormonal regulator of circulating IGF-I (3), nutritional status can also profoundly affect serum IGF-I (4). Dietary zinc (Zn) has been shown to have a strong influence on serum IGF-I concentration in humans (5–8) and rats (9,10). Matsui and Yamaguchi (11) have shown that the anabolic effects of IGF-I in osteoblastic-like cells are mediated and enhanced by Zn. Zn is also a known constituent of ∼300 enzymes, including those essential for bone metabolism such as bone alkaline phosphatase (EC 3.1.3.1) (12).

Cu is another trace mineral important for bone metabolism. Cu is a cofactor for lysyl oxidase (EC 1.4.3.13), one of the principal enzymes involved in collagen cross-linking (13). Cu deficiency has been shown to result in reduced bone strength in rats (14,15) and osteoporotic lesions in other animals (16). In humans, skeletal disorders such as osteopenia have been noted in Menke's syndrome, a genetic Cu deficiency disease (17).

An antagonistic effect of high Zn intake on Cu absorption and status has been demonstrated in rats (18) and humans (19); however, our understanding of the postabsorption interaction of these two minerals, especially the effects on accrual of bone mass and on bone quality, is very limited.

The objectives of this study were to determine the effects of graded intakes of dietary Cu and Zn on serum IGF-I concentration and on bone composition and strength in growing rats.

MATERIALS AND METHODS

Animals and diets. Weanling, male Sprague-Dawley rats (n = 84; weight, 35–40 g; Sasco Labs, Omaha, NE) were housed in individually suspended stainless steel cages. A 12-h light/dark cycle was used with lights on at 0600. The rats were randomly assigned in a 3 × 4 factorial design to 12 groups of 7 rats each, with equal mean body weights. The rats were fed modified AIN-93G basal diets (20), containing casein as the source of protein and cornstarch and sucrose as the sources of carbohydrates, for 6 wk. The diets were designed to...


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5 Abbreviations used: IGF-I, insulin-like growth factor-I; IGFBP, insulin-like growth factor-I binding protein; N, Newtons; P, phosphorus; Zn, zinc;
contain various amounts of Zn (5.0, 15 and 45 μg/g diet) and Cu (0.3, 3.0 and 10 μg/g diet), both added as the carbonate salts. The corresponding analyzed values were 0.25 ± 0.01, 3.16 ± 0.07 and 10.11 ± 0.19 for Cu and 5.16 ± 0.30, 13.53 ± 0.60 and 45.39 ± 1.30 for Zn, respectively. Because low Zn and Cu deficiency, for each group with low Zn (5.0 μg/g) intake, a group of rats (n = 7) was pair-fed, using a diet that matched the Cu concentration but was high in Zn (45 μg/g). All rats received deionized water. Except for the pair-fed groups, all other rats had free access to food. All experimental procedures conformed to the National Institutes of Health, U.S. Public Health Service and Animal Welfare Act guidelines for the ethical treatment of animals (21).

The rats were food deprived overnight, anesthetized by intraperitoneal injection of xylazine (Xylajax; Phoenix Pharmaceuticals, St. Joseph, MO) and ketamine (Ketaset; Fort Dodge Animal Health, IA) for blood collection from the descending vena cava and subsequently killed by cardiac puncture. Whole blood samples, with EDTA as the anticoagulant, were analyzed for hemoglobin with a Coulter Counter.

Liver Cu and Zn. Liver plasma Cu and Zn were determined by environmental samples. Liver plasma Cu was determined with an enzyme immunoassay kit (Biomedical Technologies, Stoughton, MA) after extraction by a modified method (22). Briefly, duplicate aliquots (10 mg) of the liver powder were decalcified in 0.5 mol of EDTA/L buffer, pH 6.2, for 18 h at 4°C, in the presence of a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN). The samples were then centrifuged, and the supernatant was diluted (1:2,000) with the sample buffer provided in the kit. The liver Cu and Zn were analyzed using a microwave digestion apparatus (Retch Mixer Mill, Type MM200; Retch, Haan, Germany). The freeze-dried extracts were reconstituted in sample buffer (1:3 dilution) provided by the enzyme immunoassay kit and analyzed for Cu concentration (Diagnostic Systems Laboratory, Webster, TX).

Previsouly described colorimetric methods were used for the determination of femur hexosamines (23) and hydroxyproline (24). Femur nitrogen concentration was determined by chemiluminescence (Pyro-chemiluminescent Nitrogen System; Antek Instruments, Houston, TX).

Physical and biomechanical analyses. For density and biomechanical measurements, the adhering muscle tissue was removed immediately before the testing to prevent desiccation of the bone samples. Right femur and lumbar vertebrae (L4) bone densities were determined by the Archimedes principle. Briefly, each bone sample was degassed in Super Q water, at room temperature, in a desiccator under vacuum for 90 min. The bones were weighed while submerged in Super Q water and then weighed out of water (weight in air). The volumetric density was calculated as previously described (25,26). Femur breaking force was determined at room temperature by a three-point bending test using a custom-made apparatus. This apparatus was interfaced with a computer and consisted of two supports (fulcrum) and a movable crosshead driven by a step motor. The distance between the fulcra was kept constant for each bone. The rate of deformation was set at 5 mm/min. The increase in force corresponding to incremental downward movement of the crosshead (0.0169 μm) was measured with a load cell and an electronic scale that was installed beneath the supports. Force-strain curves were graphed with deformation distance (mm) on the x-axis and force (N) on the y-axis. Femur breaking force was defined as the maximum force needed to break the femurs.

The geometry of the broken femurs was measured with digital calipers and a microscope (×40 magnification). Total cross-sectional and medullary areas were calculated using the elliptical cross-section model (27). Cortical thickness was calculated by averaging the four measurements of cortical thickness at the top, bottom, and sides. The cortical area was calculated by subtraction of the medullary area from the total cross-sectional area of the femur. The ultimate stress (N/mm²) was calculated by dividing the breaking force by the cortical area of the femur at the point of fracture.

Statistics. Data were evaluated by analysis of variance for the main effects of dietary Cu and Zn and their interaction in a 3 × 4 factorial design (28). Statistical significance level was set at P ≤ 0.05. Tukey’s contrasts were used for pairwise comparisons with adjustment of P-values for multiple comparisons. Simple linear and multiple regression analyses were used to determine the extent of associations between serum IGF-I and biochemical (bone IGF-I, Ca) as well as functional measurements in bone. Unless otherwise noted, data are reported as means ± pooled SD.

RESULTS

Body weights. Dietary Zn was the only determinant of final body weights, which were the lowest in the groups fed the low Zn diets (290 ± 29 g) compared with those fed the high Zn diets (315 ± 29 g; P < 0.001). Marginal dietary Zn did not adversely affect final body weights (315 ± 29 g). The final body weights in the pair-fed groups (298 ± 29 g) were not significantly different from those of the groups fed the low Zn diets, indicating that the lower body weights in the low Zn group were due to reduced food intake.

Liver Cu and Zn. Low dietary Cu significantly reduced liver Cu concentration by about 75% compared with the rats fed the marginal and high Cu diets (0.61 and 0.250 μmol/g of dry tissue; ± 0.028, respectively; P < 0.0001). The only detectable effect of dietary Zn on liver Cu was seen in the pair-fed rats (fed high Zn diets), which had slightly lower liver Cu concentration than those fed the marginal Zn diets (0.173 μmol/g of dry tissue; ± 0.0001).
Effects of graded intakes of copper (Cu) and Zinc (Zn) on serum and tibia insulin-like growth factor I (IGF-I) concentration and on femur dry weight, density, breaking strength and ultimate stress in growing male Sprague-Dawley rats

### Table 1

<table>
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<tr>
<th>Dietary treatment</th>
<th>Cu (µg/g)</th>
<th>Zn (µg/L)</th>
<th>Serum IGF-I (ng/mg of dry bone)</th>
<th>Tibia IGF-I (g)</th>
<th>Femur dry weight (g)</th>
<th>Femur cortical area (mm²)</th>
<th>Femur density (kg/L)</th>
<th>Femur breaking force (N)</th>
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ANOVA<sup>2,3</sup>

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1 Values are means; n = 7 per group. Statistical significance at P < 0.05.

2 A group of rats was pair-fed to the rats fed the low Zn diets. The Zn concentration of the diets for the pair-fed rats was 45 mg/kg of diet, and the Cu concentration matched the diet of the rats in each of the low Zn groups. Data for the pair-fed groups were included in the ANOVA, but are not shown because no differences were detected between the pair-fed groups.

3 Tukey’s pairwise contrasts are reported when a significant interaction between Cu and Zn was detected. Values within a column with a common superscript are not different based on pairwise Tukey’s contrasts (P ≥ 0.05).

**FIGURE 1** Serum insulin-like growth factor I (IGF-I) in growing male Sprague-Dawley rats fed graded intake (low, marginal and high) of Cu and Zn for 6 wk. Because the effect of dietary Zn was not significant (P = 0.09), only the effects of dietary Cu are shown. Bars assigned a different superscript are different from each other (P < 0.001). Data are means ± pooled sd, n = 21.

Cu intake was the main determinant of femur density, statistically significant interaction of dietary Zn and Cu was detected on serum IGF-I concentration, it may be of biological importance that the highest concentrations of serum IGF-I were observed in the group fed the diet containing high amounts of both Cu and Zn (1,050.1 µgL/L; Table 1).

Both dietary Cu and the interaction of Cu and Zn determined tibia IGF-I concentrations (P < 0.0001; Table 1). Opposite to serum IGF-I, tibia IGF-I concentration was the highest (by 62%) in the rats fed the low Cu diets and decreased as Cu intake increased to marginal and high intakes (from 4.92 to 4.11 to 3.04 ng/mg of dry bone, respectively; P < 0.0001). In the rats fed the marginal Cu, bone IGF-I was lower when dietary Zn was high (P < 0.0001). However, in the rats fed the high Cu diets, bone IGF-I was the highest when dietary Zn intake was also high (P < 0.05) (Table 1).

**Long bone analyses.** Dietary Cu and Zn, but not their interaction, affected various aspects of femur size and geometry. Compared with the rats fed the high Cu or the high Zn diets, femur dry weights were ~8% and ~4% lower in the rats fed the low Cu or low Zn diets, respectively (Table 1). Total cross-sectional areas of the femurs were only affected by dietary Zn and were the lowest in the rats fed the low Zn diets compared with those fed the high Zn diets (9.8 and 10.7 mm, respectively; P < 0.02; data not shown). Femur cortical area was lowest when dietary Zn was low and increased as the amount of Zn in the diet increased (P = 0.0038). Femur cortical area was also affected by a significant interaction between Cu and Zn such that this variable was the highest when the intakes of both minerals were high and the lowest when a high dietary level of one was combined with a low dietary level of the other mineral (P = 0.0151; Table 1). Diet did not affect cortical and medullary widths or the medullary area of the femurs (data not shown).

Cu intake was the main determinant of femur density,
Effects of graded intakes of copper (Cu) and zinc (Zn) on femur mineral and organic matrix composition and tibia osteocalcin concentration in growing male Sprague-Dawley rats

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<th>Dietary treatment</th>
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<th>Femur Zn μmol/g dry wt</th>
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ANOVA<sup>2,3</sup>

Pooled SD 0.21 0.11 0.003 0.25 0.28 0.009 2.7 0.018
Cu <0.0001 <0.0226 <0.0001 0.25 0.28 <0.0001 NS NS NS NS
Zn NS NS NS NS NS NS NS NS NS NS
Cu × Zn 0.021 0.0103 0.0023 NS NS NS NS NS NS 0.0030

<sup>1</sup> Values are means; n = 7 per dietary group. Statistical significance at P < 0.05.
<sup>2</sup> A group of rats was pair-fed to the rats fed the low Zn diets. The Zn concentration of the diets for the pair-fed rats was 45 mg/kg diet, and the Cu concentration matched the diet of the rats in the each of the low Zn groups. Data for the pair-fed groups were included in the ANOVA but are not shown because no differences were detected between the pair-fed groups except for femur Zn, which was significantly lower in the rats fed the low Zn diet than the pair-fed counterparts.
<sup>3</sup> Tukey’s pairwise contrasts are reported when a significant interaction between Cu and Zn was detected. Values within a column with a common superscript are not different based on pairwise Tukey’s contrasts (P > 0.05).
Effects of graded intakes of copper (Cu) and zinc (Zn) on the density and mineral composition of lumbar vertebrae in growing male Sprague-Dawley rats

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ANOVA

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<td>&lt;0.0001</td>
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1 Values are means; n = 7 per dietary group. Statistical significance at P < 0.05.
2 A group of rats was pair-fed to the rats fed the low Zn diets. The Zn concentration of the diets for the pair-fed rats was 45 mg/kg diet, and the Cu concentration matched the diet of the rats in each of the low Zn groups. Data for the pair-fed groups were included in the ANOVA but are not shown because no differences were detected between the pair-fed group and the low Zn groups.
3 Tukey’s pairwise contrasts are reported when a significant interaction between Cu and Zn was detected. Values within a column with a common superscript are not different based on pairwise Tukey’s contrasts (P ≥ 0.05).

In the groups fed the low Cu diets (P = 0.0120 and P = 0.0025, respectively) (Table 3). Vertebral Zn concentration was the lowest when dietary Zn was low and highest when dietary Cu intake was the highest (by >70%; P < 0.0001). High dietary Cu reduced the accumulation of Zn in the spinal bones as vertebral Zn concentration was higher when dietary Cu was low compared with when it was marginal and high (Table 3).

**Regression analyses.** In stepwise regression analyses that included dietary Zn and Cu and body weight in the model, serum IGF-I concentration was a strong predictor of femur breaking force and explained ~34% of the variability in breaking strength (R² = 0.34, P < 0.0001) (Fig. 3). However, a combination of femur density and body weight was the strongest predictor and accounted for more than half of the variability in femur breaking force (R² = 0.52, P < 0.0001). Femur density alone was a stronger predictor of bone strength as indicated by the partial R² (designated as R²p) (R²p = 0.31, P < 0.0001) compared with body weight alone (R²p = 0.21, P < 0.0001). Although we found a significant statistical correlation between serum IGF-I concentration and femur Ca, this relationship was very weak (R² = 0.06, P = 0.02) and is possibly of little biological significance because femur Ca also did not correlate at all with femur strength nor femur density.

**DISCUSSION**

The most remarkable finding of this study was that in the presence of graded intakes of Cu and Zn, in amounts that included a range of intakes as may be practically observed in the human population, Cu intake was the main determinant of both serum IGF-I and bone strength. Low Cu intake during rapid growth, even for the short duration of 6 wk, caused a significant reduction in the circulating levels of serum IGF-I (~30%) and in the breaking strength of long bones. In contrast, bone IGF-I concentration was increased in response to osamine concentrations were also affected only by dietary Cu and were the highest in the rats fed the low Cu diets compared with the high Cu groups (34.8 and 31.5 mmol/g of dry bone, respectively; P = 0.0004) (Table 2). Similarly, femur hydroxyproline concentration was higher in the low Cu group (0.171, and 0.166, respectively; P = 0.0412) (Table 2).

Although dietary Cu intake was the main determinant of most of the bone variables, several components of bone matrix were also affected by dietary Zn and/or the interaction of dietary Zn and Cu. Femur hydroxyproline concentration was higher with marginal Zn intake compared with low Zn intake (Table 3). Tibia osteocalcin, an osteoblast product and one of the major noncollagenous protein in the bone matrix, tended to be the lowest at extreme intakes of Cu and Zn and the highest with marginal intakes of these minerals, especially marginal intake of Cu (Table 2).

The ratio of Ca to nitrogen (Ca/N), used as a crude indicator of the proportion of the mineral components compared with the organic matrix of the femurs, was affected only by dietary Cu and was the lowest in the rats fed the low Cu diets (5.9) compared with the marginal and low Cu groups (6.2; P = 0.05) (data not shown).

**Lumbar vertebrae analyses.** Vertebral dry weights were ~23% lower in the rats fed the low Cu and low Zn compared with those fed the high levels of both minerals (Table 3). Regardless of Zn intake, vertebral bone density was lower when the rats were fed the low Cu diets than when dietary Cu was high. However, because of a significant interaction between Cu and Zn, vertebral bone density tended to be higher in rats fed diets that combined marginal to high amounts of Cu with low to marginal amounts of Zn (P < 0.0001). Similar to the long bones, the Ca and P concentrations of the vertebrae (L1–4) were affected only by dietary Cu and were the highest...
IGF-I does not necessarily match its bioactivity because some of its binding proteins (not measured in this study) potentiate IGF-I activity (e.g., IGFBP-3 and IGFBP-5) and some inhibit it (e.g., IGFBP-1 and IGFBP-4). For example, it has been shown that in starvation, IGFBP-I increases and binds IGF-I more avidly, thus inhibiting IGF-I bioactivity (4).

The mechanism by which the bones are weakened in Cu deficiency is not clear, however, decreased lysyl oxidase activity (32) leading to reduced cross-linkage in bone collagen (33) has been cited as a possible mechanism. Previous studies have shown that despite the decrease in collagen cross-linkage, the total amount of collagen is increased in the hearts of Cu-deficient rats. In the current study, we demonstrated that a similar increase in collagen concentration (as indicated by hydroxyproline measurements) occurred in the long bones of growing rats. In addition to increases in total collagen and nitrogen (a general indicator of the organic constituents of bone), femur Ca concentration was also higher in the rats fed the low Cu diets. The seemingly paradoxical finding of reduced mechanical strength but increased bone collagen concentration with low Cu intake is in agreement with previous studies (14,15). Also, noncollagenous components of long bones, as indicated by hexosamines (a measure of the glucosamine and galactosamine moieties of the glycosaminoglycans of the bone matrix), osteocalcin (an osteoblast product) and IGF-I, were also altered with low Cu intake. These collective findings of increased bone material, but reduced bone strength suggest an unsuccessful compensatory response by bone to Cu as a limiting nutrient.

In the current study, the presence of graded intakes of Cu complicated the interpretation of the results observed with dietary Zn. Our findings suggested that despite reduced growth (decreased body weight, reduced bone size), femur ultimate stress measurements were increased with low dietary Zn. This apparent increase in bone strength, without changes in bone density or composition, is difficult to explain and may be an artifact of dividing a direct measurement (breaking force), which was not affected by dietary Zn, by a calculated value (cortical area) that was not only reduced by low dietary Zn but also affected by the interaction of dietary Cu and Zn. However, this observation is intriguing and should not be dismissed because very few studies have examined the role of dietary Zn on specific biomechanical properties of bone. In a recent study, a decreased bone strength, but increased elasticity, has been reported in rats fed lower amounts of dietary Zn (1 μg/g) than in the current study (30). An adverse effect of increased dietary Zn on bone strength has only been reported with inadequate dietary Ca (34).

The antagonistic effects of Zn and Cu have been previously reported in animals and humans (35). However, information regarding the skeletal effects of the interaction between these two minerals is limited. In the current study, although long bones responded most consistently to changes in dietary Cu, the vertebrae were more sensitive to changes in both dietary Cu and Zn. The highest vertebral densities were achieved when both dietary Cu and Zn were high, implying an absence of antagonism and a possible synergy between these nutrients at high intakes. An antagonistic effect of vertebral density, however, was evident when the intake of one nutrient was low to marginal and the intake of the other nutrient was high. These findings indicate that adequate Cu intake may be more crucial for acquisition of peak bone mass in long bones, whereas adequate and balanced intakes of both Cu and Zn may be more critical for an optimal bone mass in the primarily trabecular bones of the spine.

In summary, previous studies have attributed the adverse changes in bone quality with low Cu intake primarily to low Cu intake, implying that systemic concentrations of IGF-I (primarily synthesized by the liver) are more influential in the determination of bone strength than the local production of IGF-I by osteoblasts. Zn intake primarily influenced body weight and certain aspects of bone geometry, whereas Cu intake was the main determinant of bone composition and quality. Compared with the long bones (primarily composed of cortical bones), the effects of Zn and the interaction of Cu and Zn were more pronounced in the spinal bones (primarily trabecular bone).

In humans, serum IGF-I has been shown to be positively correlated with bone mass (29) and negatively with fractures (1). The dramatic reduction in serum IGF-I in response to low dietary Cu (even with marginal intakes) and the strong association of serum IGF-I with bone breaking force in the current study imply that long term exposure to low and marginal intakes of Cu may adversely affect peak bone mass accretion during growth and thus increase the risk of osteoporosis later in life. Although in the current study the effect of dietary Zn on serum IGF-I did not reach statistical significance (P = 0.09), previous studies have reported an strong association between Zn intake and serum IGF-I in humans (5–8) and in rats fed much lower dietary concentrations of Zn (1 μg/g) (30).

Because of the paracrine and autocrine functions of IGF-I (31), we also measured bone IGF-I concentration. Bone IGF-I was the highest in the rats fed the lowest Cu diets and lowest in those fed the high Cu. This increase in bone IGF-I concentration, despite a systemic reduction of this peptide, may indicate a localized compensatory response by the bone. Although the significance of this differential response between the bone tissue and serum IGF-I is not clear, this divergence does indicate that systemic changes in IGF-I do not necessarily reflect localized changes in the target tissues and that caution must be exercised when interpreting changes in serum IGF-I.

It is possible that regulatory factors that affect hepatic synthesis differ from those that control tissue production at other sites (including bones). This may lead to differential systemic versus local tissue responsiveness, as seen in the present study, to various interventions. In addition, the level of measured IGF-I does not necessarily match its bioactivity because some
impaired maturation of bone collagen (14,36–38). In the present study, we demonstrated that the impaired bone quality associated with low Cu intake is caused not only with alterations in both the collagen and noncollagenous matrices of bone but also by a systemic reduction of circulating IGF-I. Inadequate Cu intake compromised the quality of the growing bones despite many localized compensatory alterations in the bone matrix. Because a reduction in serum IGF-I with advancing age is implicated in the etiology of osteoporosis (31), future studies aimed at characterizing the effects of these important trace minerals on the IGF-I axis proteins and functional indices of bone health are needed.

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LITERATURE CITED