Changes in dietary zinc and copper affect zinc-status indicators of postmenopausal women, notably, extracellular superoxide dismutase and amyloid precursor proteins1–4

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ABSTRACT

Background: Zinc is an essential trace element for human health and well-being; however, methods currently available for the assessment of zinc status in humans are unsatisfactory.

Objective: The objective was to critically evaluate the use of various indicators of zinc status in humans in a controlled metabolic ward study.

Design: Indicators of zinc status were measured in 25 healthy postmenopausal women aged 64.9 ± 6.7 y. After a 10-d equilibration period, volunteers consumed a diet with either a low (1 mg/d; n = 12) or a high (3 mg/d; n = 13) copper content based on a total energy content of 8.4 MJ. They received the same amount of copper throughout the study. Both groups were fed the basal diet (3 mg Zn/d) with no zinc supplement for one 90-d period, and the diet supplemented with 50 mg Zn/d for another 90-d period.

Results: Zinc supplementation significantly increased (P < 0.0001) extracellular but not erythrocyte superoxide dismutase activity. This increase was more apparent when subjects were fed the low-copper diet. Zinc supplementation in combination with the low-copper diet significantly decreased (P < 0.01) amyloid precursor protein expression in platelets. Other indicators of zinc status that were significantly elevated after zinc supplementation were as follows: plasma zinc and free thyroxine concentrations and mononuclear 5′-nucleotidase activity.


KEY WORDS Zinc, copper, extracellular superoxide dismutase activity, amyloid precursor protein, 5′-nucleotidase activity, postmenopausal women

INTRODUCTION

The essentiality of zinc for human health and well-being is well established and the consequences of severe zinc deficiency have been documented in several populations worldwide (1, 2). Conversely, a chronic excess intake of zinc is capable of interfering with the uptake and metabolism of other trace elements, notably iron and copper (3). Studies designed to examine the consequences of mild deficiency or excess zinc intake and to establish an optimal range of intakes have been impaired because methods currently available for the assessment of zinc status in humans are unsatisfactory. The most widely used approach for the assessment of zinc status has been the determination of circulating concentrations of either serum or plasma zinc. However, many factors not directly related to zinc nutriture affect this measurement (4–8).

Various investigators have suggested that zinc in blood cells, either leukocytes or erythrocytes, may more accurately reflect tissue zinc. A positive correlation between the zinc content of peripheral blood leukocytes and muscle zinc was reported to occur in healthy men (9), pregnant women (10), and patients with liver disease (11). Prasad et al (12) noted a reduction in the zinc content of peripheral blood leukocytes in humans fed a zinc-deficient diet for 6–7 mo. These values returned to normal after 2 mo of zinc repletion. During a critical evaluation of the methods used, Milne et al (13) found that platelets contributed ≈70% of the zinc found in the buffy coat. They developed a procedure to clearly separate platelets, mononucleated cells, and polymorphonuclear cells for trace metal analysis of a single blood sample. When the various methods were compared (14), the apparent zinc content of the mononucleated cells was related to the degree of contamination by platelets.

The primary metabolic role of zinc is as a component of > 200 different enzymes. Determination of the serum and tissue activities of several zinc metalloenzymes has been proposed to assess zinc nutriture. Zinc-containing enzymes that have been shown to be affected by dietary zinc in animal studies include alkaline phosphatase (15), extracellular superoxide dismutase (16), 5′-nucleotidase (17), carbonate dehydratase (15), nucleoside phosphorylase (18), and peptidyl-dipeptidase A (19). However, no significant...
effects of dietary zinc on the activities of these enzymes have been shown in carefully controlled metabolic ward studies with either men (20) or women (21). Furthermore, other studies have suggested that functional tests, such as changes in ethanol metabolism (22), are more sensitive to zinc status than are many static biochemical tests of circulating concentrations of zinc or zinc enzymes. Thus, the purpose of this study was to critically evaluate the use of various indicators of zinc status in humans in a controlled metabolic ward study. A second objective was to determine the effects of varying intakes of zinc at different amounts of dietary copper, with zinc-copper ratios ranging from 1 to > 50 on copper, iron, and lipid nutrition; this will be addressed in another paper.

SUBJECTS AND METHODS

Healthy postmenopausal women were admitted to the study after they had been informed in detail, both orally and in writing, of the nature of the research and the associated risks and after medical, psychological, and nutritional evaluations had established that they had no underlying disease and were emotionally suited for the project. Protocols were approved by the Institutional Review Boards of the University of North Dakota and the US Department of Agriculture and followed the guidelines of the Department of Health and Human Services and the Helsinki Declaration regarding the use of human subjects.

The study was conducted at 2 different times because the metabolic ward could only accommodate 14 subjects at one time. The 25 subjects who completed the study were between the ages of 50 and 76 y (\(\bar{x} \pm SD: 64.9 \pm 6.7\) y) at entry. Subjects had a mean (\(\pm SD\)) height of 159.6 \(\pm 7.6\) cm and a mean weight of 65.1 \(\pm 6.7\) kg at the beginning of the study. All of the subjects were white.

The women were maintained in a metabolic ward under close supervision for 200 d. The environment and subject management were as described previously (23). Subjects were fed a constant weighed basal diet of conventional foods that was low in copper (0.6 mg, 0.61 \(\pm 0.07\) mg by analysis) and zinc (3 mg, 2.96 \(\pm 0.24\) mg by analysis), based on an energy content of 8.4 MJ, with a 3-d menu rotation (Table 1). The diet was supplemented with 492 mg Ca (as calcium carbonate), 23 mg Fe (as ferrous sulfate), 180 mg Mg (as magnesium gluconate), and 10 \(\mu\)g cholecalciferol. Iron was supplemented to compensate for losses through phlebotomy. The diet was adequate in all other known nutrients. The subjects participated in an equilibration period of 10 d in which they received the basal diet supplemented with 1.4 mg Cu (2 mg total) and 6 mg Zn (9 mg total). The women were then randomly divided into 2 groups: 1 group (n = 12) was fed the basal diet supplemented with 0.4 mg Cu and the other group (n = 13) was fed the basal diet supplemented with an additional 2.4 mg Cu/d. The remaining 190 d was divided into two 90-d dietary periods for both groups: the basal diet with no zinc supplement was fed for one 90-d period and the basal diet supplemented with 50 mg Zn/d was fed for the other 90-d period. All of the women were fed the low-zinc diet first. The two 90-d periods were separated by a second equilibration period of 10 d, during which the basal diet supplemented with 1.4 mg Cu/d and 6 mg Zn/d was fed. Zinc was supplemented as zinc gluconate and copper was supplemented as cupric sulfate in beverages served at breakfast, lunch, and dinner. All other aspects of the diet remained constant throughout the study.

All food was weighed and provided by the research center. Foods were weighed to an accuracy of 1% during preparation in the metabolic kitchen and eaten quantitatively by the subjects. The dietary intake of each subject was based on energy needs,

<table>
<thead>
<tr>
<th>Meal</th>
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<td>Breakfast</td>
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<td>Lunch</td>
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<td>Grapefruit juice</td>
<td>Apricot nectar</td>
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<td>Chicken chef’s salad</td>
<td>Ham croissant</td>
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<td>Cranberry cherry juice</td>
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<td>Vanilla frosting</td>
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Table 1

Contents of the low-zinc and low-copper diets

The diet supplied the following per 8.4 MJ (2000 kcal): 48 g protein, 291 g carbohydrate, 77 g fat, 283 mg Ca, 14 mg Fe, 1754 mg K, 134 mg Mg, 1.4 mg Mn, 3824 mg Na, 877 mg P, 175 mg vitamin C, 1.4 mg thiamine, 1.1 mg riboflavin, 23 mg niacin, 1.9 mg vitamin B-6, 303 \(\mu\)g folate, 2.0 \(\mu\)g vitamin B-12, 1525 retinol equivalents vitamin A, 1 \(\mu\)g vitamin D, and 8 mg vitamin E. The diet was supplemented with 492 mg Ca (as calcium carbonate), 23 mg Fe (as ferrous sulfate), 180 mg Mg (as magnesium gluconate), and 10 \(\mu\)g vitamin D.
as calculated by the Harris-Benedict equation (24), plus an additional 60% of basal energy expenditure for normal activity. So that individual body weights were maintained, energy intakes were adjusted to maintain the body weight to within 2% of admission weight by adjusting the amount of the basal diet in 0.84-MJ (200-kcal) increments by proportionally changing the amounts of all foods.

Blood was drawn into plastic syringes from an antecubital vein that had been made visible by temporary use of a tourniquet, after the subjects had fasted overnight for 12 h. Aliquots were mixed with appropriate anticoagulants and processed within 90 min of the time the blood was drawn. Blood cells, platelets, mononucleated cells, neutrophils, and red blood cells were separated by a Percoll gradient (13). Blood was drawn during each of the equilibration periods and twice monthly during the dietary periods for the analysis of plasma zinc and copper and monthly during the dietary periods for the remaining analysis.

Zinc concentrations of plasma and blood cellular components were determined by flame atomic absorption spectrometry (Perkin-Elmer Corporation, Norwalk, CT). Superoxide dismutase activity in erythrocytes and serum was measured by inhibition of pyrogallol autooxidation (25). Plasma and erythrocyte alkaline phosphatase activities were determined spectrophotometrically (26). Bone-specific alkaline phosphatase activity was determined by using a monoclonal antibody (Alphase-B; Metra Laboratories, Abbott Park, IL). 5′-Nucleotidase activity in plasma, mononuclear cells, and erythrocytes was determined by the rate of nicotinamide-dineine dinucleotide formation in an enzymatic method reported by Arkestein (27). Free and total triiodothyronine, free and total thyroxine, and thyroid-stimulating hormone (TSH) were determined by radioimmunoassay (Abbott Laboratories, Abbott Park, IL).

Amyloid precursor protein (APP) expression in platelets was determined by Western blot analysis. Platelets suspended in Tyrode’s buffer (136.9 mmol NaCl/L, 2.68 mmol KC/L, 11.9 mmol NaHCO₃/L, 0.42 mmol NaH₂PO₄/L; pH 6.4) were disrupted with a tissue sonicator and combined with Laemmli sample buffer (62.5 mmol tris-HCl/L, pH 6.8; 25% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue, and 350 mmol dithiothreitol/L) and heated at 37°C for 45 min. Samples (5 μg total protein) were electrophoresed on 8.5% polyacrylamide gels in the presence of sodium dodecyl sulfate. After electrophoresis, gels and polyvinylidene fluoride blotting membranes (Immobilin-P; Millipore Corp, Bedford, MA) were equilibrated with transfer buffer (12 mmol tris/L, 96 mmol glycine/L, and 20% methanol) for 30 min. Gels were tranblotted for 40 min at 26 V. After transfer, nonspecific binding of primary antibody was blocked with 5% nonfat dry milk in tris-buffered saline (10 mmol tris/L and 150 mmol NaCl/L; pH 8.0). Blocking buffer was decanted and blots were incubated for 3 h at room temperature with primary antibody (Anti-Alzheimer precursor protein A4, clone 22C11; Boehringer Mannheim, Indianapolis). Chicken anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Pierce, Rockford, IL) was used as the secondary antibody. Membranes were incubated for 1 min in a 1:1 solution of ECL Western blot detection reagents (Amersham Life Science, Arlington Heights, IL) and then exposed to X-ray film. The bands corresponding to the 110-kDa protein were quantified with a densitometer.

Alcohol tolerance tests were performed at the end of the first equilibration period and at the end of the low- and high-zinc periods as described by Milne et al (21). One-half gram of 95% ethanol/kg body wt was mixed with 8 parts orange juice (4 mL/kg body wt) and ingested within 10 min by the volunteer after an overnight fast. This would translate to 27.5 g 95% ethanol or 26.1 g absolute ethanol for a 55-kg subject. Blood was sampled from an indwelling polytetrafluoroethylene catheter in an antecubital vein before alcohol ingestion, at 15-min intervals for the first 2 h and at 30-min intervals for the next 2 h. Blood ethanol was determined by using a commercial diagnostic kit (Sigma Diagnostics, St Louis) according to a modified enzymatic procedure (22). Serum alcohol dehydrogenase activity was determined with a method described by Shepard et al (28).

The data were analyzed by a two-way (dietary zinc and copper) repeated-measures analysis of variance (ANOVA) with the SAS general linear model program (version 6.12; SAS Institute, Cary, NC). Two of the volunteers fed low-copper diets were supplemented before the end of the 90-d dietary periods with dietary copper because of significant changes detected by the Holter electrocardiograph; these volunteers were not included in the statistical analysis. Tukey’s contrasts were used to differentiate among means for variables that had been significantly affected by the treatments. Variance in the data were expressed as pooled SDs, calculated as the square root of the mean square error from the ANOVA.

RESULTS

The measured indicators of zinc status in this study were variably affected by dietary zinc and copper (Table 2). Plasma zinc concentrations were significantly lower and platelet zinc concentrations tended to be lower, but not significantly so, when subjects were fed 3 mg Zn/d than when their diets were supplemented with 53 mg Zn/d. Plasma zinc concentrations did not change significantly from equilibration values during the low-zinc period; however, plasma zinc concentrations were significantly elevated from equilibration values beginning 1 mo after zinc supplementation and remained elevated throughout the zinc-supplementation period. There were no significant changes in plasma zinc concentrations between 1 and 3 mo of zinc supplementation. Plasma zinc concentrations were within the normal range for healthy adults (10.7–18.4 μmol/L) throughout the low-zinc period. In contrast, 8 of 23 volunteers had plasma zinc concentrations > 18.4 μmol/L at the end of the zinc-supplementation period. Neither erythrocyte nor erythrocyte membrane zinc concentrations responded significantly to changes in dietary zinc.

Zinc supplementation significantly increased bone-specific alkaline phosphatase activity (0.40 ± 0.02 compared with 0.32 ± 0.02 μkat/L) and seemed to increase plasma alkaline phosphatase activity (1.66 ± 0.02 compared with 1.57 ± 0.02 μkat/L); however, this difference was not significant. Erythrocyte membrane alkaline phosphatase activity did not change significantly with the different dietary treatments. Zinc supplementation significantly increased mononuclear white cell 5′-nucleotidase activity (17.90 ± 1.54 compared with 12.16 ± 1.54 U/L) and significantly decreased plasma 5′-nucleotidase activity (4.64 ± 0.19 compared with 6.36 ± 0.19 U/L). A significant interaction between copper and zinc affected mononuclear 5′-nucleotidase activity such that the difference caused by zinc supplementation was apparent when subjects were fed the high-copper diet but not when they were fed the low-copper diet. Changes in dietary zinc did not significantly affect red blood cell membrane 5′-nucleotidase activity.
Extracellular, but not erythrocyte, superoxide dismutase activity was significantly increased by zinc supplementation. This effect was more apparent when subjects were fed the low-copper diet. The different responses of extracellular and erythrocyte superoxide dismutase activities to alterations in zinc and copper intakes were magnified when expressed as the percentage change from the first equilibrium values (Figure 1). In women fed the low-copper diet, extracellular superoxide dismutase activity was elevated when the diet was supplemented with zinc. In contrast, in women fed the low-copper diet, erythrocyte superoxide dismutase activity was lower than equilibrium values during the low dietary zinc period and was depressed even further during the

![Figure 1](https://academic.oup.com/ajcn/article-abstract/71/3/781/4729197/784 DA VIS ET AL)
zinc-supplementation period. Similarly, in women fed the high-copper diet, extracellular superoxide dismutase activity was higher during the high-zinc period than during the low-zinc period; however, erythrocyte superoxide dismutase activity was lower during the high-zinc period than during the low-zinc period. These data suggest that zinc supplementation increases extracellular superoxide dismutase activity but depresses erythrocyte superoxide dismutase activity.

Dietary zinc influenced circulating thyroid hormone concentrations (Table 2). Plasma free thyroxine concentrations were significantly higher and plasma free triiodothyronine concentrations tended to be higher, but not significantly so, during the high-zinc period than during the low-zinc period. However, dietary zinc did not significantly affect total thyroxine or triiodothyronine concentrations.

APP expression in platelets was significantly \( (P < 0.01) \) affected by both dietary zinc and copper (Figure 2). Zinc supplementation significantly depressed platelet APP expression only when subjects were fed the low-copper diet. However, the data for APP expression was analyzed only from the second group of subjects because samples from the first group of subjects had not yet been analyzed and were lost when a flood occurred. Therefore, because of the small sample size (only 4 subjects consumed the low-copper diet), these results need to be confirmed in future studies.

Ethanol metabolism was not significantly affected by zinc intake (Figure 3). There were no significant differences in the rate of clearance of ethanol or in alcohol dehydrogenase activity among the different dietary treatments.

**DISCUSSION**

This study critically evaluated the use of various indicators of zinc status in humans in a controlled metabolic ward study. As observed in previous studies, plasma zinc concentrations and mononuclear 5'-nucleotidase activities seem to be sensitive to alterations in dietary zinc (17, 29). Furthermore, bone-specific alkaline phosphatase apparently is a more sensitive indicator of zinc status than is the measurement of total alkaline phosphatase activity in plasma. However, our results indicate that 2 potential new indicators of zinc status—extracellular superoxide dismutase activity and platelet APP expression—may also respond to changes in zinc intake.

When subjects were fed low dietary zinc, extracellular superoxide dismutase activity and plasma zinc concentrations were significantly lower than when subjects were supplemented with zinc. The differences between these 2 groups were magnified when subjects were fed the low-copper diet. Paik et al (30) reported that extracellular superoxide dismutase activity was significantly lower in subjects with low serum zinc concentrations. Furthermore, Olin et al (16) observed that dietary zinc affected extracellular superoxide dismutase activity in both rats and rhesus macaques, and DiSilvestro (31) observed that extracellular superoxide dismutase activity in rats was reduced by marginal or deficient copper intakes as well as by turpentine-induced inflammation. Thus, the results obtained support the concept that the assessment of serum extracellular superoxide dismutase activity is of value as an indicator of zinc status in humans. However, its activity is also affected by dietary copper.

Although extracellular superoxide dismutase and Cu/Zn superoxide dismutase are similar with respect to catalytic function and their dependence on zinc and copper, no similarities are apparent in amino acid composition and antigenic properties (32). Furthermore, the 2 enzymes respond differently to alterations in dietary zinc. Whereas the activity of extracellular superoxide dismutase increased when subjects were supplemented with zinc, the activity of Cu/Zn superoxide dismutase was decreased by supplemental zinc. The reduction in erythrocyte

**FIGURE 2.** Mean (±SEM) platelet amyloid precursor protein expression in postmenopausal women after alterations in dietary zinc and copper for 90 d. *Significantly different from all other dietary treatments, \( P < 0.05 \) (ANOVA followed by Tukey’s contrasts).
Cu/Zn superoxide dismutase activity was consistent with the observations of Fischer et al. (33) and suggests possible adverse effects of long-term zinc supplementation. The elevation of serum zinc concentrations above the normal range in 8 of 23 volunteers during the zinc-supplementation period also suggests that zinc supplementation could have adverse effects.

Despite the lower activity of extracellular superoxide dismutase than of erythrocyte Cu/Zn superoxide dismutase, it is an important scavenger of free radicals. In vitro experiments suggest that free radicals may contribute to atherogenesis. Wang et al. (34) found that plasma extracellular superoxide dismutase concentrations were significantly lower in patients with a history of myocardial infarction than in control subjects. Thus, depressed extracellular superoxide dismutase activity may have functional consequences because extracellular superoxide dismutase may be protective against the risk of coronary heart disease.

Future work investigating the usefulness of extracellular superoxide dismutase as an indicator of zinc status must take into account 2 considerations. First, this enzyme activity would not be a useful zinc-status indicator for certain subjects, such as persons with renal disease, liver disease, and diabetes, who tend to have higher than normal extracellular superoxide dismutase concentrations (35). Furthermore, the assay must be sensitive enough to distinguish normal from low values. In the current study, the changes in activity observed with zinc supplementation were rather small and plasma zinc appeared to be a more sensitive zinc-status indicator than did extracellular superoxide dismutase activity.

Another potential indicator of zinc status, copper status, or both is platelet APP expression. In the current study, platelet APP expression was significantly reduced when subjects were fed the low-copper diet supplemented with zinc. Previous investigators showed that APP binds zinc and copper at distinct sites and that the binding of zinc and copper to APP is suggested to control APP conformation and stability (36, 37). APP is a precursor protein for the β-amyloid 4 protein, which is the major protein component isolated from the amyloid deposits of Alzheimer patients. APP has been found to reduce copper (II) to copper (I) by an electron transfer reaction and thus contribute to the aggregation and toxicity of β-amyloid 4 protein (38). Furthermore, in an in vitro study, Bush et al. (39) observed that zinc ions can cause the β-amyloid 4 protein to form clumps resembling the amyloid plaques found in the brains of Alzheimer disease patients. Previous studies showed that patients with Alzheimer disease have depressed temporal lobe zinc concentrations (40), elevated cerebrospinal fluid zinc and copper concentrations (41, 42), elevated hepatic zinc concentrations with decreased zinc bound to metallothionein (43), and depressed concentrations of astrocytic growth inhibitory factor, a metallothionein-like protein that chelates zinc (44). In a group of nuns who received no supplemental zinc, serum zinc concentrations were found to have moderate-to-strong negative correlations with senile plaque and neurofibrillary tangle counts in each of 7 brain regions (45). Therefore, there may be an abnormality in the uptake or distribution of zinc in the brain of patients with Alzheimer disease. Thus, these results suggest that alterations in dietary zinc and copper may have implications for the pathogenesis of Alzheimer disease.

Another potential effect of alterations in zinc intake is a change in alcohol metabolism. Das et al. (46) noted that the clearance of alcohol from the blood of severely zinc-deficient rats was impaired. This impairment correlated with decreased activity of liver alcohol dehydrogenase, a zinc-containing enzyme. Ethanol metabolism also was significantly affected in postmenopausal women after they consumed a diet containing 2.6 mg Zn/d for 4 mo (21). The increased intestinal uptake of ethanol and lower concentration-dependent blood clearance rates observed at the end of the low dietary zinc period were corrected within 1 mo of zinc supplementation. In the current study, alcohol metabolism was not sensitive to changes in zinc intake. One possible explanation for the different results obtained in the 2 studies is that in the current study subjects were fed 3 mg Zn/d for 90 d but in the previous study (21) subjects were fed 2.6 mg Zn/d for 141 d. Furthermore, in a study of young men (47), apparent ethanol
absorption was not affected significantly by consumption of 1, 2, 3, or 4 mg Zn/d for 21 d. These results suggest that changes in dietary zinc do not have large effects on alcohol metabolism. However, the effects of zinc intake on ethanol metabolism may be better clarified by analyzing urinary as well as blood measurements.

In conclusion, our results clearly showed that the measurement of serum extracellular superoxide dismutase activity may be useful as a marker of the functional assessment of zinc status in humans; however, it must be emphasized that the activity of this enzyme can be influenced by other dietary factors, such as copper intake. APP expression is also apparently influenced by dietary zinc and copper; however, this finding needs to be confirmed in a larger group of subjects.

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