Zinc Supplementation Does Not Alter Sensitive Biomarkers of Copper Status in Healthy Boys1–4

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Abstract
The Tolerable Upper Intake Levels (UL) for zinc for children were based on limited data and there is concern that the UL may be set too low. The first effect of excessive zinc intake is a reduction in copper status. The primary objective of this study was to examine the effect of zinc supplementation on copper status in children. Healthy, 6- to 8-y-old boys from Ontario, Canada were assigned to take a placebo (n = 10) or 5 mg (n = 10), 10 mg (n = 9), or 15 mg (n = 8) of zinc supplement daily for 4 mo in a double-blinded, placebo-controlled, randomized trial. Biochemical measures were evaluated at baseline and after 2 and 4 mo of supplementation. Food records were completed near the baseline and 4-mo visits. Age and anthropometric measurements did not differ (P > 0.05) between treatment groups at baseline. Mean zinc intakes from food alone (10.9–14.8 mg zinc/d) approached or exceeded the UL of 12 mg/d. Compared with the placebo group, the zinc groups had a greater change in the urine zinc:creatinine ratio at 4 mo (P = 0.02). Traditional (plasma copper and ceruloplasmin activity) and more sensitive biomarkers of copper status, including erythrocyte SOD1 activity and the erythrocyte CCS:SOD1 protein ratio, were unchanged in zinc-supplemented boys, demonstrating that copper status was not depressed. Serum lipid measures and hemoglobin concentrations were also unaffected and gastrointestinal symptoms were not reported. These data provide evidence in support of the need for reexamining the current UL for zinc for children. J. Nutr. 143: 284–289, 2013.

Introduction
The Tolerable Upper Intake Levels (UL)10 for zinc for infants, children, and adolescents (established by the Institute of Medicine) were based on one study that reported no depression in copper status or other adverse health effects in healthy, full-term infants fed formula containing 5.8 mg zinc/L. (1,2).

A no-observed-adverse-effect-level of 4.5 mg/d was derived and used to set the UL for 0- to 6-mo-old infants at 4 mg/d (2). This UL was adjusted upwards for older infants, children, and adolescents aged 7–12 mo (5 mg/d), 1–3 y (7 mg/d), 4–8 y (12 mg/d), 9–13 y (23 mg/d), and 14–18 y (34 mg/d) on the basis of relative body weight (2).

Presently, a large proportion of young children in North America have zinc intakes exceeding the UL (3–5). A recent cross-sectional survey of a national random sample of United States children showed that zinc intakes from food, beverages, and supplements exceeded the UL for 68% of infants, 47% of toddlers, and 72% of preschoolers (4). The lack of recognized zinc toxicity (6) in children from routine monitoring has raised suspicions that the UL for zinc may be set too low for children.

Given the paucity of data on which the UL were based, more studies are needed to define amounts of zinc intake that are safe and harmful for children that can be used to reexamine the UL. Depression of copper status is the first effect of excessive zinc intake. Thus, the value of these studies largely depends on the ability to detect mild reductions in copper status. Most zinc supplementation studies in children conducted to date did not examine effects on copper status or assessed status using...
insensitive indicators such as plasma copper concentration (7). It is possible that reductions in copper status were missed from lack of monitoring or use of insensitive biomarkers.

In erythrocytes and other cell types, copper chaperone for copper-zinc superoxide dismutase (CCS) protein is more stable when copper is scarce and its content increases (8–13). In rats, an increase in erythrocyte CCS (eCCS) was shown to be a more sensitive indicator of zinc-induced copper deficiency compared with traditional biomarkers of copper status and erythrocyte copper-zinc superoxide dismutase (eSOD1) activity (13), the indicator used to set the UL for zinc for adults (2,14,15). Because eCCS increases and eSOD1 decreases with copper deficiency, an increase in the eCCS:SOD1 protein ratio can be used as an indicator of a reduction in copper status (10). The primary objective of this study was to determine the effect of zinc supplementation on copper status in healthy boys using traditional and more sensitive indicators of copper status, including the eCCS:SOD1 ratio. Effects on lipid profile, iron status, and gastrointestinal health were secondary outcome measures.

Participants and Methods

Study design. This prospective study was a randomized, parallel trial. Participants, caregivers, and investigators were unaware of the treatment interventions. Participants were assigned to receive 2 chewable tablets daily (one in the morning with breakfast and one in the evening with dinner) for a 4-mo period. Tablets were placebo or zinc supplements providing a total of 5 mg (Zn5), 10 mg (Zn10), or 15 mg (Zn15) zinc/d. Supplementation began a mean of 3.8 ± 0.3 d after the initial screening visit (baseline). If either the morning or evening dose was missed, participants were instructed to take 2 tablets the following time. If 2 or more doses were missed, participants were to continue the dosing regimen as usual. Jamieson Laboratories prepared and masked (with a letter code) the placebo and zinc tablets. The code was concealed until all data were ready for statistical analyses. The zinc and placebo tablets were visually indistinguishable and contained microcrystalline cellulose, dicalcium phosphate dihydrate, croscarmellose sodium, and magnesium stearate. Zinc (as zinc gluconate) was added to this formulation at the expense of microcrystalline cellulose. The certificate of analysis indicated that the placebo tablets contained 0.110 mg zinc/tablet and the zinc tablets contained the indicated amounts of zinc. Placebo and zinc tablets contained <0.0002 mg copper/tablet and <0.0063 mg iron/tablet. The zinc and copper contents in samples of placebo and zinc tablets were verified analytically by the investigators. Zinc tablets were approved by the Natural Health Products Directorate, Health Canada [zinc 2.5 mg: natural product number (NPN) 80001389; zinc 5 mg: NPN 80001392; and zinc 7.5 mg: NPN 80001393].

Permuted blocks of 4 were used for randomization of participants to treatment groups. Participants were assigned to a treatment group immediately upon enrolment in the study. Participants and caregivers were provided with a log sheet to record supplement use and encourage compliance. They were also instructed to record any adverse effects (e.g., nausea, vomiting, abdominal cramps). This study was approved by the Research Ethics Boards of Health Canada (REB 2004–0057) and the University of Guelph (REB 05JA018). Prior to the start of the study, caregivers of the participants signed an informed consent document and participants gave their assent to partake. Participants were treated in accordance with the ethical standards of the Health Canada and University of Guelph Research Ethics Boards on human experimentation.

Subjects. Boys from Guelph, Ontario, Canada and surrounding regions were enrolled in the study from February 2007 to May 2010. After the study began, the protocol was amended in an effort to increase recruitment efficiency (approved January 3, 2008); the age range for participants was expanded from 7–8 y to 6–8 y. Participants were considered eligible if they were 6–8 y of age, had not taken a mineral supplement within the past 3 mo, and agreed to not take mineral supplements, sodium fluoride, or aspirin during the study. Normal baseline results for routine blood and urine tests including complete blood count, electrolytes, and indicators of renal and hepatic function were a prerequisite for participation (Supplemental Table 1). Boys were excluded if they had chronic medical conditions.

Compliance. Each participant was given a vial containing 80 placebo or zinc tablets. Every month, the vial was collected in exchange for a new vial. In total, 4 vials (numbered 1 to 4) were given to each participant in sequential order. Percent compliance was calculated by dividing the sum of the tablets missing from vials by the number of tablets to be consumed. Compliance for the first 2 mo of the study was determined by calculating compliance from vials 1 and 2. Compliance from 2 to 4 mo was determined by calculating compliance from vials 3 and 4.

Anthropometric measurements. Height and weight measurements were recorded at each visit. Height was measured to the nearest 0.1 cm with a Seca Road Rod 214 portable stadiometer. Body weight was measured with a digital scale accurate to 50 g (Lifesource MD, Rosscraft). BMI was derived from height and weight measurements. Height-for-age Z-scores, weight-for-age Z-scores (WAZs), and BMI-for-age Z-scores were calculated in comparison with the 2000 CDC growth charts for the United States (16,17). The Cornell University age calculator was used for age calculations (18).

Blood and urine samples. Blood and urine samples were collected at baseline and after −2 (mean: 66.9 ± 0.8 d) and 4 mo (mean: 126.7 ± 1.0 d) of supplementation. Venous blood samples were collected in the morning at a LifeLabs clinic after an overnight fast. A topical anesthetic (EMLA Patch) was applied prior to the blood draws. Participants were to collect a mid-stream urine sample at home the morning of the visit and bring it with them to the clinic. Blood samples were collected for determination of complete blood count and blood chemistry tests by LifeLabs (Supplemental Table 1). An additional blood sample was collected in a BD Vacutainer Trace Element K2 EDTA tube for isolation of plasma and erythrocytes. Briefly, samples were centrifuged (1000 × g, 15 min, 4°C) to separate the blood into its components. Plasma was collected; the white buffy coat containing mononuclear cells and platelets was removed and discarded. The remaining erythrocytes were washed 3 times with cold isotonic saline [0.9% (wt:v) NaCl]. Aliquots of plasma and erythrocytes were frozen at −80°C until analysis. Baseline and follow-up analyses at 2 and 4 mo for each biochemical measurement (excluding tests performed by LifeLabs) of a specific participant were completed within the same analytical run.

Mineral analyses. The zinc concentration in plasma and urine was measured by flame atomic absorption spectroscopy using an AAnalyst 400 spectrometer (PerkinElmer). Copper in plasma was measured by graphite furnace atomic absorption spectroscopy using an AAnalyst 600 spectrometer (PerkinElmer). Plasma was diluted 1:10 and 1:20 with deionized water prior to measurement of zinc and copper, respectively. Zinc and copper concentrations were determined relative to standard curves generated using National Institute of Standards and Technology-certified atomic absorption standards (SCP Science). Certified reference materials from different sources were analyzed alongside samples to verify analytical accuracy. Each sample was measured in triplicate (zinc) or duplicate (copper) and the mean reported.

Assays. Plasma ceruloplasmin activity was determined using o-dianisidine dihydrochloride as previously described (19). Each sample was analyzed in duplicate and the mean reported. SOD1 activity in erythrocyte lysates was measured using a commercial Superoxide Dismutase Assay kit (item no. 706002, Cayman Chemical). SOD1 activity was normalized to hemoglobin concentration. The hemoglobin concentration was determined with Drabkin’s Reagent (Sigma) using a microplate assay. Urine creatinine concentration was measured using VITROS CREA Slides with a Vitros 5,1 FS by Ortho-Clinical Diagnostics (Johnson and Johnson).
**Western blotting.** Erythrocytes (−20 μL packed cells) were lysed with 400 μL of Tris buffer, pH 7.2 containing a protease inhibitor cocktail (Roche). The lysate was centrifuged (1000 × g, 5 min, 4°C) and the supernatant was recovered. Erythrocyte lysates (40 μg hemoglobin) were resolved over 8–16% Tris-glycine gradient gels (Invitrogen) under denaturing and reducing conditions. All samples were analyzed simultaneously using multiple gels. Proteins from all gels were transferred onto a single polyvinylidene difluoride membrane using a Genie Blotter (Idea Scientific). Membranes were blocked in Tris buffered saline (TBS)-TWEEN [20 mmol/L Tris, 500 mmol/L NaCl, 0.1% (v/v) Tween 20, pH 7.5] supplemented with 5% (wt:v) nonfat dry milk (BioRad) for 30 min at room temperature. The membrane was probed overnight at 4°C with an anti-CCS antibody (H7, Santa Cruz Biotechnology) at a 1:500 dilution in TBS-TWEEN supplemented with 0.5% (wt:v) nonfat dry milk. The membrane was washed with TBS-TWEEN and probed with an anti-mouse HRP-conjugated secondary antibody at a 1:2500 dilution for 2 h at room temperature. After washing, the membrane was soaked in SuperSignal West Dura Extended Duration Technology (Pierce) at a 1:500 dilution in TBS-TWEEN supplemented with 0.5% (wt:v) nonfat dry milk. The membrane was washed with TBS-TWEEN and probed with an anti-mouse HRP-conjugated secondary antibody at a 1:2500 dilution for 2 h at room temperature. Next, the membrane was washed with TBS-TWEEN and probed with an anti-mouse HRP-conjugated secondary antibody at a 1:2500 dilution for 2 h at room temperature. Finally, the membrane was washed with TBS-TWEEN and probed with an anti-mouse HRP-conjugated secondary antibody at a 1:2500 dilution for 2 h at room temperature. For this experiment, the signal was detected using enhanced chemiluminescence using a CHEMI Genius® Bio Imaging System (Syngene). CCS signal intensity was quantified using Scion Image software. The membrane was stripped with stripping buffer [62.5 mmol/L Tris-HCl pH 6.8, 2% (wt:v) SDS, 100 mmol/L 2-mercaptoethanol] for 30 min at 53°C. SOD1 was detected using an SOD1 antibody (FL154, Santa Cruz Biotechnology) at a 1:500 dilution as described for CCS.

**Dietary intake.** Three-day food records were completed near the baseline and 4-mo visits. Caregivers were asked to record all foods, including beverages, consumed by the participants for 3 consecutive days. Food records included 1 weekend day and 2 weekdays. Detailed instructions were provided to the caregivers. Scales were provided to weigh meat. Dietary data were entered into the Nutrition Survey System (Food Directorate, Health Canada). Information on energy and nutrient consumption for each participant was generated using the 2005 Canadian Nutrient File.

**Statistical analyses.** The number of participants required to detect a reduction in copper status was calculated based on the zinc supplementation study in women used by the Institute of Medicine to establish the UL for zinc for adults (15). A formula to estimate sample sizes for comparing the means from 2 independent groups was used. To detect a 47% reduction in eSOD1 activity (that was reported in that study after 10 wk of zinc supplementation) with 90% power and an α value of 0.05, 6 participants/group would be needed. Based on data from rat studies (10,13), to detect a doubling of the eCCS:SOD1 protein ratio or eCCS protein (normalized to GAPDH), 5 and 8 participants/group would be required, respectively.

Baseline values for biochemical and anthropometric data were analyzed using an ANOVA F-test with a main effect for treatment group. Changes from baseline at 2 and 4 mo were compared with ANCOVA with baseline values as a covariate to reduce the effect of differences at baseline. When overall results were significant, the Tukey-Kramer test was used to determine which groups differed. For changes from baseline, combined data from all zinc groups were compared with the placebo group using a linear contrast for ANCOVA with baseline values as a covariate.

Differences in energy and nutrient intakes from food between treatment groups at baseline and 4 mo were evaluated using an ANOVA model with a main effect for treatment group and assessed with Armitage’s test for trend. Data were reported as the mean ± SEM or least-squares mean ± SEM. Significance was set at P < 0.05. Data were analyzed using SAS 9.2 (SAS Institute) and SigmaPlot 11.2 (Systat Software).

**Results**

**Subject characteristics.** Thirty-nine participants were enrolled in the study. Thirty-seven completed the study. Two participants dropped out of the study prior to the 2-mo visit. One participant (Zn10 group) refused the blood draw and one participant (Zn15 group) did not want to continue taking the supplement. Data from these 2 participants were excluded from the analyses. One participant from the Zn5 group had a low white blood cell count and low neutrophil and lymphocyte counts at the initial screening visit. One participant from the Zn15 group had a low neutrophil count. Both of these participants repeated the baseline visit at a later time, had normal results, and were enrolled in the study. Age and anthropometric measurements did not differ between treatment groups at baseline (P > 0.05) (Table 1).

**Compliance.** Mean compliances for the first 2 mo of the study were 91.2 ± 3.5% (placebo), 79.8 ± 6.5% (Zn5), 80.5 ± 4.0% (Zn10), and 80.9 ± 3.5% (Zn15). Mean compliances from 2 to 4 mo were 86.3 ± 5.0% (placebo), 75.6 ± 9.1% (Zn5), 71.3 ± 6.7% (Zn10), and 68.5 ± 9.1% (Zn15). For the full study, 86% of the participants complied >60% of the time.

**Dietary intake.** Intakes of zinc, copper, and iron from food were compared between groups (Table 2). Mean intakes of zinc, copper, and iron exceeded the RDA for 4- to 8-y-olds of 5, 0.440, and 10 mg/d, respectively. Mean zinc intakes from food approached or exceeded the UL of 12 mg/d. Energy intake and percent energy contribution from carbohydrates, lipids, and proteins were similar between groups at baseline and 4 mo (P > 0.05) (Supplemental Table 2).

**Biochemical measures.** Zinc groups had a larger change in urine zinc:creatinine ratio compared with the placebo group at 4 mo (P < 0.02) (Table 3). The plasma copper concentration and ceruloplasmin activity were higher in the Zn5 group compared with the Zn15 group at baseline (P < 0.05) (Table 3). Because ~90% of plasma copper is bound to ceruloplasmin, this result is likely explained by the random assignment of participants with normally higher ceruloplasmin to the Zn5 group. The changes in plasma copper concentration, plasma ceruloplasmin activity, eSOD1 activity, and eCCS:SOD1 protein ratio at 2 and 4 mo did not differ between groups (P > 0.05) (Table 3). The changes in

### TABLE 1 Age and anthropometric measurements of boys at baseline

<table>
<thead>
<tr>
<th>Subjects, n</th>
<th>Placebo</th>
<th>Zn5</th>
<th>Zn10</th>
<th>Zn15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mo</td>
<td>95.7 ± 3.0</td>
<td>92.7 ± 3.4</td>
<td>96.7 ± 2.5</td>
<td>94.8 ± 3.2</td>
</tr>
<tr>
<td>Height, cm</td>
<td>133.0 ± 1.9</td>
<td>130.3 ± 1.6</td>
<td>129.5 ± 2.3</td>
<td>129.4 ± 1.5</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>28.7 ± 1.6</td>
<td>30.1 ± 2.5</td>
<td>29.7 ± 3.2</td>
<td>27.4 ± 1.5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>16.1 ± 0.5</td>
<td>17.7 ± 1.3</td>
<td>17.5 ± 1.4</td>
<td>16.3 ± 0.7</td>
</tr>
<tr>
<td>Height-for-age Z-score</td>
<td>0.83 ± 0.22</td>
<td>0.77 ± 0.18</td>
<td>0.23 ± 0.32</td>
<td>0.41 ± 0.23</td>
</tr>
<tr>
<td>WAZ</td>
<td>0.54 ± 0.25</td>
<td>0.80 ± 0.40</td>
<td>0.43 ± 0.40</td>
<td>0.36 ± 0.33</td>
</tr>
<tr>
<td>BMI-for-age Z-score</td>
<td>0.00 ± 0.31</td>
<td>0.38 ± 0.50</td>
<td>0.36 ± 0.36</td>
<td>0.14 ± 0.34</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM. WAZ, weight-for-age Z-score; Zn5, 5 mg zinc supplement group; Zn10, 10 mg zinc supplement group; Zn15, 15 mg zinc supplement group.
serum lipid measures and hemoglobin concentrations also did not differ between groups \((P > 0.05)\) (Supplemental Table 4).

**Discussion**

The goal of this study was to contribute to stronger evidence on which to base the UL for zinc for children. Zinc supplementation of healthy boys with doses ranging from modest to high did not alter the plasma copper concentration, plasma ceruloplasmin activity, eSOD1 activity, or eCCS:SOD1 protein ratio, strongly suggesting that copper status was not depressed. This result is consistent with most zinc supplementation studies in children and infants \((1,20–24)\). It is important to note, however, that in many previous studies, copper status was assessed using insensitive biomarkers. In the present study, more sensitive indicators were used, including eSOD1 activity and eCCS protein content. For this study, we chose to express eCCS relative to eSOD1 content \((i.e., \text{ eCCS:SOD1 ratio})\). Because eSOD1 protein decreases with copper deficiency, normalizing eCCS to eSOD1 has the advantage of potentially magnifying the eCCS response if the copper deficiency is severe enough to induce a reduction in eSOD1 \((10)\).

**TABLE 3** Biomarkers of zinc and copper status of boys at baseline and change after 2 and 4 mo of supplementation with zinc or placebo\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Zn5</th>
<th>Zn10</th>
<th>Zn15</th>
<th>Effect of treatment</th>
<th>Zinc groups vs. placebo(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma zinc, (\mu\text{mol/L})</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>15.2 ± 0.7</td>
<td>14.5 ± 0.7</td>
<td>14.4 ± 37</td>
<td>15.0 ± 0.8</td>
<td>NS(^3)</td>
<td></td>
</tr>
<tr>
<td>(\Delta) 2 mo</td>
<td>−0.5 ± 0.7</td>
<td>−0.1 ± 0.7</td>
<td>−0.2 ± 0.7</td>
<td>0.4 ± 0.8</td>
<td>NS(^4)</td>
<td>NS</td>
</tr>
<tr>
<td>(\Delta) 4 mo</td>
<td>−0.6 ± 0.6</td>
<td>−0.4 ± 0.6</td>
<td>−0.2 ± 0.6</td>
<td>0.4 ± 0.6</td>
<td>NS(^4)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Urine zinc, (\mu\text{g/mmol CR})</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>79 ± 8</td>
<td>56 ± 6</td>
<td>66 ± 8</td>
<td>84 ± 9</td>
<td>0.07(^5)</td>
<td></td>
</tr>
<tr>
<td>(\Delta) 2 mo</td>
<td>−9 ± 8</td>
<td>−3 ± 9</td>
<td>−1 ± 9</td>
<td>6 ± 9</td>
<td>NS(^4)</td>
<td>NS</td>
</tr>
<tr>
<td>(\Delta) 4 mo</td>
<td>−16 ± 7</td>
<td>4 ± 7</td>
<td>12 ± 8</td>
<td>−2 ± 8</td>
<td>0.08(^4)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Plasma copper, (\mu\text{mol/L})</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>19.0 ± 0.7(^ab)</td>
<td>22.4 ± 0.8(^a)</td>
<td>19.0 ± 1.3(^ab)</td>
<td>18.4 ± 0.9(^b)</td>
<td>0.02(^3)</td>
<td></td>
</tr>
<tr>
<td>(\Delta) 2 mo</td>
<td>0.8 ± 0.8</td>
<td>1.1 ± 0.9</td>
<td>0.1 ± 0.8</td>
<td>−0.7 ± 0.9</td>
<td>NS(^4)</td>
<td>NS</td>
</tr>
<tr>
<td>(\Delta) 4 mo</td>
<td>0.5 ± 0.8</td>
<td>−0.3 ± 0.9</td>
<td>0.2 ± 0.8</td>
<td>−0.1 ± 0.9</td>
<td>NS(^4)</td>
<td>NS</td>
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<tr>
<td><strong>Plasma Cr, units/L</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>138 ± 8(^a)</td>
<td>170 ± 7(^a)</td>
<td>146 ± 13(^ab)</td>
<td>134 ± 6(^b)</td>
<td>0.03(^3)</td>
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<tr>
<td>(\Delta) 2 mo</td>
<td>7 ± 8</td>
<td>12 ± 9</td>
<td>2 ± 8</td>
<td>−5 ± 9</td>
<td>NS(^4)</td>
<td>NS</td>
</tr>
<tr>
<td>(\Delta) 4 mo</td>
<td>12 ± 7</td>
<td>6 ± 8</td>
<td>4 ± 8</td>
<td>6 ± 8</td>
<td>NS(^4)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>eSOD1 activity, units/g Hb</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>9280 ± 690</td>
<td>9020 ± 360</td>
<td>8750 ± 460</td>
<td>8270 ± 550</td>
<td>NS(^3)</td>
<td></td>
</tr>
<tr>
<td>(\Delta) 2 mo</td>
<td>−120 ± 500</td>
<td>−490 ± 500</td>
<td>870 ± 520</td>
<td>−70 ± 560</td>
<td>NS(^3)</td>
<td>NS</td>
</tr>
<tr>
<td>(\Delta) 4 mo</td>
<td>−150 ± 440</td>
<td>−50 ± 440</td>
<td>−740 ± 460</td>
<td>100 ± 490</td>
<td>NS(^3)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>eCCS:SOD1 ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline(^6)</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>NS(^3)</td>
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</tr>
<tr>
<td>(\Delta) 2 mo</td>
<td>0.05 ± 0.05</td>
<td>−0.04 ± 0.05</td>
<td>−0.02 ± 0.05</td>
<td>−0.13 ± 0.06</td>
<td>NS(^4)</td>
<td>0.08</td>
</tr>
<tr>
<td>(\Delta) 4 mo</td>
<td>−0.05 ± 0.07</td>
<td>0.02 ± 0.07</td>
<td>0.08 ± 0.07</td>
<td>−0.02 ± 0.08</td>
<td>NS(^4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\) Values are mean ± SEM (baseline) or least-squares mean ± SEM (change values). Means in a row without a common letter differ, \(P < 0.05\) (Tukey-Kramer test). NS, \(P > 0.1\).

\(^2\) Linear contrast for ANCOVA with baseline values as a covariate.

\(^3\) ANCOVA F-test.

\(^4\) ANCOVA with baseline values as a covariate.

\(^5\) Baseline eCCS:SOD1 ratio was arbitrarily set to 1 for all subjects.

\(^6\) Copper, ceruloplasmin; Cr, creatinine; eCCS:SOD1, erythrocyte CCS:SOD1; eSOD1, erythrocyte SOD1; Zn5, 5 mg zinc supplement group; Zn10, 10 mg zinc supplement group; Zn15, 15 mg zinc supplement group.
Copper binding to CCS promotes its degradation by the 26 S proteasome (11,12). When copper is limiting, CCS is more stable and its content increases. Given that mature erythrocytes are anucleate and lack much of the protein degradation capability, regulation of CCS expression by copper may occur during erythrocyte maturation. Thus, it was important that this study be of adequate length to allow sufficient turnover of erythrocytes. Because the lifespan of erythrocytes in humans is ~120 d, a 4-mo supplementation period was chosen.

Zinc supplementation has been reported to cause a reduction of HDL cholesterol (25–27) and decreased iron absorption (28,29). In this study, zinc supplementation did not affect any serum lipid measures, including total cholesterol, LDL cholesterol, HDL cholesterol, total cholesterol:HDL ratio, and TGs. Hemoglobin concentrations were also unaffected. This result is in agreement with a recent systematic review of randomized zinc supplementation trials demonstrating no effect on hemoglobin concentrations (30). The lack of a decrease in hemoglobin or changes in other hematological measurements (data not shown) indicates that iron status was not markedly depressed. It should be noted that although a hematological profile can detect iron deficiency resulting in anemia, it does not detect reductions in iron stores. It is unlikely, however, that iron status was compromised given that copper status is more sensitive to zinc overload and all biomarkers of copper status examined were unaffected. Participants also did not report any gastrointestinal symptoms, which are sometimes reported with high doses of supplemental zinc (25).

The change in the urine zinc:creatinine ratio was larger for the zinc groups at the end of the study, demonstrating an effect on a recognized biomarker of zinc supplementation (31–33). Plasma zinc was unaffected by zinc supplementation. Studies have shown that zinc administered as a supplement between meals is more effective at increasing the plasma zinc concentration in children compared with a similar amount of zinc given in a fortified food (34,35). It has been proposed that the postabsorptive metabolism of zinc may be different in the presence of certain food components (35). In this study, zinc was administered as a supplement, but participants were specifically instructed to consume the zinc tablets with food. Other studies have shown an increase in urine zinc in the absence of an increase in plasma zinc (31,33). Urine zinc may be a more sensitive marker of zinc supplementation compared with plasma zinc in individuals without a preexisting zinc deficiency or with normal plasma zinc concentrations.

Boys receiving zinc supplements had larger weight gains and change in WAZ compared with boys receiving the placebo at mid-study. It is well established that zinc supplementation increases linear growth velocity and weight gain in zinc-deficient children with restricted growth (20,36). This effect on weight was unexpected, because baseline data indicated that participants were not zinc deficient, stunted, or underweight. Baseline plasma zinc concentrations were well above the lower cutoff value (9.9 μmol/L) suggested by the International Zinc Nutrition Consultantative Group (37) and height-for-age Z-score and WAZ for all participants were > –2 (data not shown). The observed effect on weight should be interpreted with caution, because this study was not powered to detect effects on growth and a 4-mo study is too short to assess effects on growth in children of this age range.

Estrogen has been shown to increase plasma copper and ceruloplasmin activity (38). At puberty, estrogen levels markedly increase in girls. Because some girls can experience puberty at a young age, we chose to include only boys in this study to avoid the possible confounding effects of estrogen on biomarkers of copper status. Importantly, the results of this study are expected to be generalizable to girls.

The main strength of this study was the use of sensitive biomarkers to assess effects on copper status. To our knowledge, this is the first study to use eCCS protein content to assess effects of zinc supplementation on copper status in humans. Other strengths include the dose-response design and inclusion of dietary, growth, and health data as descriptors of the participants. The enrolment of only healthy boys was important, because common childhood illnesses such as diarrhea and infections can increase zinc requirements and affect biomarkers of copper status that can confound the interpretation of the results (7). This study also enrolled children who may be at risk for zinc overload; mean zinc intakes from food at baseline were greater than the UL for all treatment groups. A limitation was the relatively small number of participants per group, although it was adequate to examine effects on copper status.

In sum, zinc supplementation of 5–15 mg/d for 4 mo did not alter traditional and more sensitive measures of copper status in healthy boys. Lipid profile and hematological measurements were also unaffected and gastrointestinal symptoms were not observed. The absence of adverse effects in this study is noteworthy. When zinc intake from food and supplement is considered, all boys who received a zinc supplement had a total zinc intake exceeding the UL and many participants in the highest dose groups had intakes more than double the UL. Despite the need for further studies using higher zinc doses to define a lowest-observed-adverse-effect-level for children, this work adds to the existing evidence base that suggests a need for reexamining the current UL for zinc for children.

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Literature Cited


