Long-term high copper intake: effects on indexes of copper status, antioxidant status, and immune function in young men[1–3]

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ABSTRACT

Background: Short-term high copper intake does not appear to affect indexes of copper status or functions related to copper status, but the effects of long-term high copper intake are unknown.

Objective: A study was conducted in men to determine the effect of long-term high copper intake on indexes of copper status, oxidant damage, and immune function.

Design: Nine men were confined to a metabolic research unit (MRU) for 18 d and were fed a 3-d rotating menu providing an average of 1.6 mg Cu/d. The men continued the study under free-living conditions for 129 d and supplemented their usual diets with 7 mg Cu/d. The men then returned to the MRU for 18 d of the same diet as during the first period, except that copper intake was 7.8 mg/d. Plasma copper, ceruloplasmin activity, ceruloplasmin protein, plasma malondialdehyde, benzylamine oxidase activity, erythrocyte superoxide dismutase, hair copper, urinary copper, and urinary thiobarbituric acid–reactive substances were measured during each MRU period.

Results: Ceruloplasmin activity, benzylamine oxidase, and superoxide dismutase were significantly higher at the end of the second MRU period than at the end of the first. Urinary copper excretion, hair copper concentrations, and urinary thiobarbituric acid–reactive substances were significantly higher during the second MRU period than during the first. Polymorphonuclear cell count, the percentage of white blood cells, lymphocyte count, and interleukin 2R were affected by copper supplementation. Antibody titer for the Beijing strain of influenza virus was significantly lower in supplemented subjects after immunization than in unsupplemented control subjects.

Conclusions: Under highly controlled conditions, long-term high copper intake results in increases in some indexes of copper status, alters an index of oxidant stress, and affects several indexes of immune function. The physiologic implications of these changes are unknown.   *Am J Clin Nutr* 2004;79:1037–44.

INTRODUCTION

Copper deficiency is associated with changes in several indexes of copper status, including the plasma copper concentration, ceruloplasmin activity, and erythrocyte superoxide dismutase (SOD) activity (1, 2). However, indexes of copper status remain unchanged over a broad range of intakes as the result of homeostatic control mechanisms (3, 4). Several studies have shown that when the copper intake is increased above the usual intake for short periods of time, status indexes are not affected (4–9). Information is not available on the effect of long-term high copper intake on indexes of copper status, however. The new dietary reference intakes for copper include a recommended dietary allowance of 0.9 mg/d and a tolerable upper intake level of 10 mg/d for adults (10).

Humans adapt to different dietary copper intakes by varying their efficiency of copper absorption. We found that when dietary copper was 0.4 mg/d, absorption was 67% (11), and when intake was 0.8 mg/d, absorption was 56%, much more efficient than when intakes were higher. For example, copper absorption was only 12% when the intake was 7.5 mg/d, but it increased to 36% when the intake was 1.7 mg/d (12). In addition to changes in the efficiency of copper absorption, the excretion of endogenous copper was low when dietary copper was low, whereas endogenous excretion increased as dietary copper increased. These adaptations in absorption and endogenous excretion help to prevent the development of copper deficiency when the copper intake is low and copper toxicity when the copper intake is high. However, total copper retention may increase as intake increases. If this increased retention continues over time, the increased intake could produce changes in copper status.

Excess copper may have a prooxidant effect by catalyzing the formation of the reactive hydroxyl radical via the Haber-Weiss reaction (13). Excess copper in rats provokes increased oxidant damage to membrane lipids and the DNA of liver and kidney tissues, as reviewed by Bremner (13). In oxidatively stressed erythrocytes, the combination of copper and vitamin C, but not

copper alone, produces increases in methemoglobin, an oxidized form of hemoglobin (14). Because copper and vitamin C are known to be a prooxidant couple in vitro (14, 15), we also determined the effect of oral vitamin C doses on measures of oxidant damage before and after copper supplementation.

Both deficiency and excessive intake of copper have been reported to reduce several aspects of immune response in animal models, including neutrophil numbers, lymphocyte proliferation, and antigen-specific antibody production (16–19). Copper deficiency or its marginal intake in humans also impairs immune cell numbers and functions (18–20). Effects of high intakes of copper on human immune response have not been previously studied.

The study described here was conducted to determine the effects of a high copper intake over a period of 5 mo on indexes of copper status, in vivo oxidant damage, and immune function. The indexes of copper status examined included plasma copper, ceruloplasmin, erythrocyte SOD, benzylamine oxidase (BAO) activity, urinary copper excretion, hair copper concentration, and markers of lipid peroxidation. The indexes of immune response examined included the number of circulating white blood cells, serum cytokines, delayed hypersensitivity skin response, and antibody response; these indexes were reported to be altered in studies with animal models.

SUBJECTS AND METHODS

Subjects

Volunteers for the study were recruited through newspaper advertisement. After screening, 11 healthy young men were selected to participate. Screening included a medical history, urine and blood tests, a test for intestinal parasites, electrocardiogram, and psychological tests. Reasons for exclusion from the study were physical signs of health impairment, weight-for-height more than ±20% of normal on the basis of the Metropolitan Life Insurance Company tables (1983), history of cardiac abnormalities or an abnormal electrocardiogram result, use of prescription medicines or chronic use of nonprescription drugs, substance abuse, use of tobacco, or apparent inability to reside in the metabolic research unit. Two subjects did not complete the study and their data are not included. The study protocol was reviewed and approved by the Human Subjects Review Committee of the University of California, Davis, and by the US Department of Agriculture Human Studies Review Committee. Details of the study and the potential risks associated with increased dietary copper were explained to the volunteers, and each signed a consent form before the beginning of the study. The subjects' profiles, including body weights at the beginning of each live-in period and average energy intakes during the live-in parts of the study, are shown in **Table 1**. Body weights did not differ significantly between the 2 live-in periods.

Experimental design

The men lived in the Western Human Nutrition Research Center's metabolic research unit for 18 d (MP-A) while consuming a diet containing 1.6 mg Cu/d, followed by a 129-d free-living period during which they consumed their usual diets along with copper supplements containing 7 mg Cu/d. They returned to the metabolic research unit for another 18 d (MP-B), and their diets were supplemented with 6.2 mg Cu/d for a total of 7.8 mg/d. They

TABLE 1
Characteristics of the volunteers[1]

	Value
Age (y)	38 ± 7
Height (cm)	175 ± 7
Body weight (kg)	
Metabolic period A	74 ± 13
Metabolic period B	76 ± 13
Energy intake (kcal/d)	2911 ± 293

[1] All values are $\bar{x}$ ± SD.

were supervised by the nursing staff at all times while living in the metabolic research unit. During the free-living period, they returned to the center to receive a supply of supplements and report any concerns, and the investigators verified compliance and monitored the men's body weights and vital signs. Body weights and vital signs were measured daily throughout the confined portions of the study. The men were required to walk 3 miles (≈5 km) twice daily under the supervision of the nursing staff to maintain their physical fitness. Occasionally, treadmill walking was substituted for the outdoor walk.

The experimental protocol is summarized in **Figure 1**. Blood samples were collected for the determination of indexes of copper status at the beginning and end of the live-in parts of the study. Complete urine and stool collections continued throughout the live-in parts of the study. Stools were collected twice during the free-living part of the study to monitor compliance with copper supplements. Hair was collected on day 15 of each 18-d live-in part of the study. On day 14 of each live-in part of the study, a 1-g dose of ascorbic acid was added to the juice consumed with the breakfast meal. Delayed hypersensitivity tests were performed on day 8 of each live-in part of the study, and influenza immunization injections were given to the subjects after 12 wk of supplementation and to 10 control subjects who were not taking copper supplements. The control subjects were within the same age range as the subjects and the weight criteria for the control subjects was the same as for the study subjects. The control subjects did not have a history of cardiac abnormalities or other chronic diseases, did not use prescription drugs, and did not have a history of substance abuse or use of tobacco.

Diet

A 3-d rotating menu was used during both live-in parts of the study. The diet supplied at least the recommended amounts of all known essential nutrients. The 3-d menu is shown in **Table 2**.
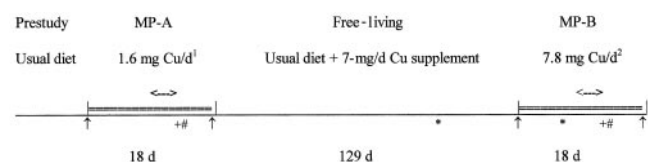

FIGURE 1. Experimental design. MP-A, metabolic period A, 1.6 mg Cu/d; MP-B, metabolic period B, 7.8 mg Cu/d; [1], 3-d rotating diet; [2], 3-d rotating diet + supplement containing 6.2 mg Cu/d; ====, urine collected for urinary copper measurement; ↔, urine collected for measurement of thiobarbituric acid–reactive substances; ↑, blood drawn for measurement of indexes of copper status and immune function; +, ascorbate load test for measurement of plasma ascorbate; #, hair collection; *, influenza vaccine administered to subjects and control subjects, followed by serum collection for antibody titres.

TABLE 2
Three-day rotating menu

	Menu day 1	Menu day 2	Menu day 3
Breakfast	Bagel with jam	Oat bran waffles	Corn-oat bran flakes cereal
	Canned pineapple	Pancake syrup	Blueberries
	Cranberry-apple juice	Apple juice	Milk, 1% fat
Lunch	Tuna and cheese sandwich	Ham sandwich	Creamy herbed chicken breast with rice and mixed vegetables
	Coleslaw	Cucumber salad	Vanilla wafers
	Milk, 1% fat	Tapioca pudding	Grape juice
		Milk, 1% fat	
Dinner	Broccoli	Turkey breast	Low-fat meatloaf and ketchup
	Chicken pasta with cream sauce	Stuffing and gravy	Garlic mashed potatoes
	Cranberry-apple juice	Green beans	Peas and carrots
		Rasberry gelatin	Canned peaches
		Apple juice	Grape juice
Snack	Orange sherbet	Apple pie	Chocolate cake with icing
All day	Safflower oil margarine	Safflower oil margarine	Safflower oil margarine
	Extra energy drink	Extra energy drink	Extra energy drink

The daily energy content of the basic diet was 9.2 MJ (2200 kcal), with ≈15–16% of energy (87 g) from protein, 55% of energy from carbohydrate, and 30% of energy from fat. The ratio of polyunsaturated to saturated fatty acids was 1.10, and the diet contained 196 mg cholesterol/d. The energy intake, shown in Table 1, was adjusted for each volunteer on the basis of body weight and diet records so that each would maintain his initial weight throughout the study. This was done by adding energy to the basic diet in the form of an extra energy drink. It contained maltodextrin, cornstarch, sugar, whipping cream, cottonseed oil, and water and had a ratio of polyunsaturated to saturated fatty acids of 1.01. If a subject gained or lost >1% of his baseline body weight, his energy intake was adjusted by removing or adding the extra energy drink in 420-kJ (100-kcal) increments. Baseline body weights were the average of the weights on days 2–4 of the study. Body weights remained relatively constant, averaging 74 kg in MP-A and 76 kg in MP-B. The maximum weight loss was 2 kg and the maximum gain was 3 kg.

The copper content of the diet was 1.6 mg/d during MP-A and 7.8 mg/d during MP-B. A solution containing copper sulfate was added to the formula drink of each meal to achieve the desired copper concentration. The solution also provided 2.7 mg Zn/d to achieve the recommended dietary allowance of zinc. The amount of copper was the only variable between MP-A and MP-B. During the free-living period, the subjects were instructed to consume supplements that provided 7 mg Cu/d as copper sulfate. The supplements were divided between the morning and evening meals. There are numerous reports of gastrointestinal effects, including abdominal cramps, nausea, diarrhea, and vomiting, of high concentrations of copper in drinking water (10). Controlled studies suggest that nausea may occur when drinking water contains >3 mg/L (21). One of our subjects experienced nausea at the beginning of the supplement period, which was alleviated when he consumed his supplemental copper after eating food.

The subjects kept dietary records for 5 d before the study and for 5 d during the free-living period. Copper intake was estimated to be an average of ≈1.6 mg/d from these records with the use of the NUTRITION DATA SYSTEM FOR RESEARCH (NDS-R), version 4.01 (Nutrition Coordinating Center, University of Minnesota, 1998).

Sample collection and processing

Precautions were taken to avoid trace element contamination during all phases of sample collection, preparation, and analysis, as described previously (22). Blood samples to be analyzed for copper were drawn into trace element–free containers. Urine was collected in 24-h pools. These were diluted with deionized water to 3000 g and acidified with a 1% volume of concentrated hydrochloric acid. Daily collections were inverted several times to insure homogeneity, and subsamples were combined into 3-d pools. Samples were frozen for later analysis of copper and thiobarbituric acid–reactive substances (TBARS). Urinary creatinine was measured in 24-h and 3-d pools to monitor completeness of urine collections. Blood was collected for health screening (complete blood counts and blood chemistry), plasma copper, ceruloplasmin concentration and activity, reduced and total plasma ascorbates, plasma malondialdehyde, BAO activity, and erythrocyte SOD activity. Additional blood samples were taken at 4 and 24 h after the 1-g vitamin C doses on days 14 of MP-A and MP-B for determination of reduced and total ascorbate. Hair was collected from an area in the back of the head about 2 cm in diameter. The hair in the area was first cut to a length of 1.5 cm and was then cut at the scalp. During MP-B, the new growth of hair in the same area was collected. Urine and plasma samples were stored at −20 °C before copper analysis. Samples collected for enzyme assays and oxidant damage were stored at −70 °C before analysis. Samples of EDTA-treated plasma for ascorbate analysis were mixed immediately after venipuncture with an equal volume of 100 g metaphosphoric acid/L (Aldrich, Milwaukee) to stabilize the ascorbate. The protein precipitate was removed by centrifugation for 10 min at 3000 × g and 4 °C and the supernatant fluid was frozen at −70 °C for later analysis.

Sample analysis

Copper analysis

Plasma was thawed and 4 mL of 6.5% trichloroacetic acid was added to 1 mL plasma. Tubes were capped, agitated in a vortex mixer for 10 s, and then centrifuged at 1860 × g for 20 min. Plasma copper was measured by flame atomic absorption spectrophotometry with a Perkin Elmer Spectrophotometer model 5100 with an AS-60 autosampler (Perkin Elmer, Norwalk, CT). A reference pool of human plasma, prepared by us, and a reagent blank were analyzed with the study samples. The copper concentration of the plasma reference pool was 12.4 ± 0.3 μmol/L, within the established concentration range for the pool. The recovery of copper standard solutions added to the plasma samples averaged 94%.

Urinary copper was measured by graphite furnace atomic absorption spectrophotometry with a Perkin Elmer HGA-600 furnace and an AS-60 autosampler (Perkin Elmer). Pyrocoated graphite tubes with L'vov platforms, deuterium-arc background correction, wavelength of 324.7 nm, high-purity argon purge gas, and peak area integration of 4 s were used for analysis. Temperature settings were 120 °C for 45 s for drying, 800 °C for 60 s for charring, and 2450 °C for 4 s for atomization. A reference pool of urine prepared by us was used for quality-control purposes. The concentration of copper in the reference pool was 3.93 ± 0.10 μg/L, within the established concentration range for the pool. The recovery of copper standard solutions added to a urine composite sample averaged 97.6%.

Hair was rinsed twice with acetone, then diethyl ether, washed with dodecyl sodium sulfate, filtered, and then washed with deionized water. It was rinsed again with acetone and then deionized water and dried at 70 °C until dry and stored in a desiccator. Thirty milligrams of hair was weighed and ashed in a muffle furnace for 16 h at 450 °C. Some hair samples were smaller than 30 mg, and in those cases the amount available was used. The ash was dissolved in 1 mol HCl/L and was analyzed by graphite furnace atomic absorption spectrophotometry under the same conditions as for analysis of urine samples. Recovery of copper standard solutions added to a composite of hair solutions averaged 106%.

Ceruloplasmin

Plasma ceruloplasmin activity was measured after the oxidation of *o*-dianisidine dihydrochloride as described by Schosinsky et al (23). All time points for each subject were assayed on the same day to minimize between-assay variability. Plasma ceruloplasmin protein concentrations were measured by using a commercial radial immunodiffusion kit (The Binding Site, Birmingham, UK). The results are expressed in mg/L plasma.

Erythrocyte superoxide dismutase

Erythrocyte SOD activity was determined by the method of Marklund and Marklund (24). Whole blood was hemolyzed and SOD was extracted with an ethanol:chloroform mixture. SOD was determined in the extracted hemolysate with correction for volume changes during the extraction process. Final erythrocyte SOD activity is expressed as units of activity per mg hemoglobin. Hemoglobin was measured with a kit using Drabkin's reagent (Sigma, St Louis).

Plasma benzylamine oxidase

Assay of plasma BAO activity was based on the method of Lizcano et al (25) and involved the catalyzed conversion of [^{14}C]benzylamine to [^{14}C]benzaldehyde. At acidic pH, benzylamine is totally insoluble in toluene, whereas benzaldehyde can be quantitatively extracted into toluene containing 0.35% dyphenayloxazole. Radioactivity of extracted benzaldehyde was measured by using a liquid scintillation counter. Specific activity was expressed in units per liter (U/L), where 1 unit enzyme converts 1 μmol substrate per minute.

Malondialdehyde

Plasma malondialdehyde was measured in EDTA-treated blood by an HPLC determination of the thiobarbituric acid–malondialdehyde adduct (26). The results are calculated from a calibration curve based on malondialdehyde generated in vivo by acid hydrolysis of 1,1,3,3-tetramethoxypropane (Sigma) and are expressed as micromolar malondialdehyde equivalents.

Urinary thiobarbituric acid–reactive substances

Urinary TBARS for the day 10–12 pool were measured by formation of the thiobarbituric acid–malondialdehyde adduct (26) and determination of the adduct concentration by fluorescence spectroscopy at an excitation wavelength of 525 nm and an emission wavelength of 547 nm. Results were calculated from a calibration curve of freshly prepared 1,1,3,3-tetraethoxypropane standards and are expressed as micromolar malondialdehyde equivalents.

Plasma vitamin C

Reduced ascorbic acid and total ascorbate [ascorbic acid plus dehydroascorbic acid (DHA)] were measured by HPLC separation and electrochemical detection of ascorbate before and after reduction with *tris*[2-carboxyethyl]phosphine hydrochloride (Pierce Chemicals, Rockford, IL; 27). Plasma DHA was then estimated indirectly by subtraction of reduced ascorbate from total ascorbate.

Immune function

A complete and differential blood cell count was performed on the EDTA-treated blood by using a Serono Baker Automated System (model 9000 Diff; Allentown, PA). Serum concentrations of interleukin 2R (IL-2R) and IL-6 were measured with enzyme-linked immunosorbent assay kits (Immunotech, Beckman Coulter, Marseille, France) and those of immunoglobulin and C3 by using a naphlometer (Auto ICS Immunochemistry, Beckman Instruments, Bear, CA). Delayed hypersensitivity skin response to a battery of 7 recall antigens (tetanus, diphtheria, streptococcus, tuberculin, candida, trichophyton, and proteus) was determined by using Multiskin test applicators. Swellings >1 mm were recorded 24 and 48 h after the application of the antigens. The sum of the mean diameter for all positive responses (mean induration) and the number of positive responses (antigen score) were determined. All study subjects, along with a control group of 10 subjects, were immunized with a trivalent (Beijing, Sydney, Harbin) influenza vaccine (Connaught Labs, Swiftwater, PA) 2 wk before the end of the high copper intake period. Blood samples were collected before and 14 d after immunization; serum antibody titers were determined by using the hemagglutination inhibition assay (28). Fold increases (postimmu-

TABLE 3
Effect of copper supplementation on indexes of copper status during metabolic periods A and B[1]

	Metabolic period A	Metabolic period B
Plasma copper (μmol/L)[2]	14.3 ± 0.5	14.9 ± 0.5
Superoxide dismutase (U/g Hb)[2]	1065 ± 47	1206 ± 24[3]
Ceruloplasmin[2]		
Activity (U/L)	107 ± 3	116 ± 5[3]
Protein (mg/L)	278 ± 7	288 ± 12
Benzylamine oxidase activity (U/L)[2]	283 ± 25	377 ± 31[3]
Urinary copper (μg/d)[4]	20.3 ± 0.8	25.6 ± 1.1[3]
Hair copper (μg/g)[5]	9.2 ± 3.1	21.1 ± 5.9[3]

[1] All values are $\bar{x}$ ± SEM; $n = 9$. Metabolic period A, 1.6 mg Cu/d; metabolic period B, 7.8 mg Cu/d. Hb, hemoglobin.

[2] Values on the last day of the metabolic period.

[3] Significantly different from metabolic period A, $P < 0.05$ (ANOVA followed by Tukey's test if a significant F was found).

[4] Average urinary copper throughout the metabolic period.

[5] Value for hair collected on day 15 of each metabolic period.

TABLE 4
Plasma ascorbate after the vitamin C dose[1]

Time point[2]	Plasma reduced ascorbate	Plasma dehydroascorbate[3]
Metabolic period A	μmol/L	%
Baseline	51.6 ± 5.3	1.52 ± 1.4
4 h	114.2 ± 8.7[4]	3.47 ± 1.71
24 h	58.7 ± 3.2	1.39 ± 1.94
Metabolic period B		
Baseline	58.7 ± 5.7	−1.80 ± 0.96
4 h	121.0 ± 9.4[4]	4.27 ± 2.38
24 h	65.8 ± 4.1	−0.63 ± 1.52

[1] All values are $\bar{x}$ ± SEM; $n = 9$. Metabolic period A, 1.6 mg Cu/d; metabolic period B, 7.8 mg Cu/d.

[2] Time points are before (baseline) and 4 and 24 h after a 1-g dose of vitamin C consumed with the breakfast meal on day 14 of each metabolic period.

[3] Values are percentage of total plasma ascorbate.

[4] Significantly different from baseline and 24 h, $P < 0.05$ (repeated-measures ANOVA adjusted for multiple comparisons by use of Scheffe's method).

nization/preimmunization titers) for the antibody titers against each of the 3 viral strains were calculated and compared between the control and high-copper groups.

Statistical analysis

Statistical analysis was performed with the personal computer version 8.2 of the STATISTICAL ANALYSIS SYSTEM (29). Descriptive statistics, including means, SDs, and plots, were tabulated and compared. PROC GLM was used to perform analysis of variance (ANOVA) on the indexes of copper status. An ANOVA model was used to determine the effects of dietary copper on ceruloplasmin activity, ceruloplasmin concentration, erythrocyte SOD activity, plasma BAO activity, plasma copper, hair copper, urinary copper, and indexes of immune function. If a significant F was found, Tukey's test was used to determine which treatment means differed. Repeated-measures ANOVA was performed to determine the effect of dietary copper intake on plasma reduced, total, and dehydroascorbate before and 4 and 24 h after the vitamin C dose. Statistical probabilities were adjusted for multiple comparisons by the use of Scheffe's method. Plasma malondialdehyde and urinary TBARS values at day 14 of MP-A were compared with values at day 14 of MP-B by the use of paired t tests. A significance level of 0.05 was used for all statistical tests.

RESULTS

Copper status

Data on the indexes of copper status are summarized in **Table 3**. Erythrocyte SOD, ceruloplasmin activity and protein, BAO activity, and plasma copper were measured twice during each metabolic period. The table shows comparisons at the ends of MP-A and MP-B, when the dietary copper intake would be expected to have had the maximum effect. Erythrocyte SOD was significantly higher at the end of MP-B than at the end of MP-A. The results for ceruloplasmin and BAO activity were similar. Neither ceruloplasmin protein nor plasma copper differed significantly between treatments. Urinary copper was measured throughout MP-A and MP-B. It was higher during MP-B than

during MP-A. Hair copper, collected once in both MP-A and MP-B, was higher during MP-B. Ceruloplasmin protein and activity, plasma copper, BAO activity, and urinary copper all differed significantly between subjects, but SOD activity did not. The range of average values by subject was 251-312 mg/L (SEM: 10.9 mg/L) for ceruloplasmin protein, 93.5-124.5 U/L (SEM: 4.6 U/L) for ceruloplasmin activity, 13.0-16.6 μmol/L (SEM: 0.35 μmol/L) for plasma copper, 224-472 U/L (SEM 28 U/L) for BAO activity, and 18.5-30.9 μg/d (SEM 3.3 μg/d) for urinary copper. The average hair copper concentration of the subjects ranged from 3 to 40 μg/g, but values were not significantly different between subjects.

Antioxidant status

The values for reduced plasma ascorbate and DHA before and 4 and 24 h after the 1-g vitamin C dose given on day 14 of each metabolic period are shown in **Table 4**. Plasma ascorbate increased significantly at 4 h postdose ($P < 0.001$), and DHA tended to increase at 4 h compared with baseline ($P < 0.06$). However, these indexes showed no relation to copper intake, nor was there a copper intake–by-time interaction. Plasma malondialdehyde was measured at the preascorbate baseline time point, and urinary TBARS were measured for the 3-d urine pool from days 10–12. As shown in **Figure 2**, urinary TBARS were significantly higher in MP- B than in MP-A ($P < 0.05$), whereas plasma malondialdehyde concentrations did not change significantly.

Immune function

As shown in **Table 5**, the number of total white blood cells did not change significantly during the course of the study; however, their composition did change. The number of neutrophils (expressed as a percentage of the total white blood cells) decreased from the start of the study to the end of the study and the percentage of neutrophils decreased ($P < 0.05$ for both). Lymphocyte numbers increased significantly ($P < 0.05$) with the increased copper intake. The number or percentage of other types of white blood cells, including monocytes, eosinophils, and basophils, did not change significantly with altered copper intake
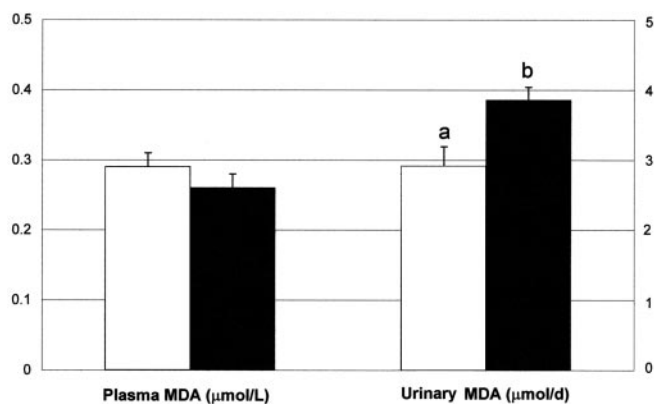
FIGURE 2. Mean (±SEM) effect of copper supplementation on plasma malondialdehyde (MDA) and urinary thiobarbituric acid–reactive substances (TBARS) during metabolic period A (MP-A; □) and metabolic period B (MP-B; ■). Copper intake was 1.6 mg/d in MP-A and 7.8 mg/d in MP-B. Plasma MDA was measured on day 14 of each metabolic period. Urinary TBARS values are means for 3-d urine collections on days 10–12 of each metabolic period. $n = 9$. Bars with different letters are significantly different, $P < 0.05$ (paired t test).

(not shown). Concentrations of IL-2R, which is associated with regulating T cell proliferation, decreased ($P < 0.05$; Table 5). Inflammatory response as determined by the serum concentration of IL-6 almost doubled; however, the results were not significant. The serum concentrations of immunoglobulin G and C3 were not altered with increased intake of copper. The anergy panel, examined 48 h after the application of the antigens, resulted in mean indurations of 9.3 ± 2.2 mm at the end of low-copper period and 11.5 ± 2.0 mm at the end of high-copper period; corresponding mean antigen scores were 1.9 ± 0.3 and 2.3 ± 0.4. Differences between the 2 periods for the induration and antigen scores were not significant, and there was no significant difference between these variables determined 24 h after the application of the antigens (data not shown).

Increases in antibody titers after immunization with the trivalent influenza vaccine for the supplemented subjects and the

TABLE 5
Effect of copper supplementation on indexes of immune function during metabolic periods A and B[1]

	Metabolic period A	Metabolic period B
White blood cells ($\times 10^9$/L)	5.9 ± 0.3	6.2 ± 0.3
Polymorphonuclear cells		
($\times 10^9$/L)	3.3 ± 0.2	3.2 ± 0.2^2
(% of WBC)	53.8 ± 1.7	50.3 ± 1.6^2
Lymphocytes		
($\times 10^9$/L)	2.14 ± 0.06	2.48 ± 0.06^2
(% of WBC)	38.2 ± 1.5	41.3 ± 1.3
Interleukin 2R (pg/mL)	33.1 ± 3.5	27.3 ± 2.2^2
Interleukin 6 (pg/mL)	1.4 ± 0.5	2.3 ± 0.6
Immunoglobulin G (mg/dL)	1134 ± 44	1130 ± 54
C3 (mg/dL)	113 ± 4	115 ± 4

[1] All values are $\bar{x} \pm$ SEM of 2 values per subject for the metabolic period, except for interleukin 6, which is based on 1 value per subject for the metabolic period. $n = 9$. Metabolic period A, 1.6 mg Cu/d; metabolic period B, 7.8 mg Cu/d.

[2] Significantly different from metabolic period A, $P < 0.05$ (ANOVA followed by Tukey's test if a significant F was found).

TABLE 6
Effect of copper supplementation on serum influenza antibody titers[1]

	Viral strain		
	Beijing	Sydney	Harben
	titre ratio		
Control subjects ($n = 10$)	47 ± 9	92 ± 55	32 ± 14
Experimental subjects ($n = 9$)	14 ± 4^2	14 ± 4	12 ± 6

[1] All values are $\bar{x} \pm$ SEM of the fold increase (postimmunization/preimmunization) in antibody titers. Control subjects consumed their usual diets without supplements. Experimental subjects consumed their usual diets and copper supplements of 7 mg/d during the free-living period.

[2] Significantly different from control subjects, $P < 0.05$ (Student's t test).

unsupplemented control subjects are shown in **Table 6**. Immunization caused a 47-fold increase in the antibody against the Beijing strain in the control subjects, whereas the increase in the experimental subjects was only 14-fold; this difference between the groups was significant ($P < 0.05$). The differences between groups in fold increases in antibody titers for the Sydney and Harbin strains were not significant, probably in part because of the large variability.

DISCUSSION

Copper status

An earlier study showed that several indexes of copper status decrease significantly during a very-low-copper diet and then increase in response to copper repletion (22). When copper-deficient individuals are supplemented with copper, deficiency symptoms are reversed and indexes of copper status improve (1, 30, 31).

Several conditions result in increases in plasma copper and ceruloplasmin. These variables also rise, often to 2 or 3 times normal concentrations, with inflammatory conditions, infectious disease, hematologic disease, diabetes, coronary and cardiovascular diseases, uremia and malignant diseases, and oral contraceptive use; during pregnancy; and after surgery (32, 33). Smoking and some drugs will also increase serum copper concentrations. Ceruloplasmin is an acute phase reactant and the rise in serum copper is primarily due to the hepatic synthesis and release of ceruloplasmin. The increases in serum copper and ceruloplasmin parallel one another (34).

In contrast with the conditions discussed above, copper supplementation studies have shown that indexes of status do not tend to be influenced by additional dietary copper. For example, copper status did not change in healthy infants as a result of copper supplementation (35). Two different studies reported no change in indexes of copper status when adult men were supplemented with 2 or 3 mg Cu/d (5, 8). In 6- and 8-wk supplementation regimens with ≤6 mg Cu/d, consistent increases in traditional indexes of copper status were not observed (6, 9). Nor did indexes of copper status change after feeding diets containing 7.8 mg Cu/d for 24 d (4).

Although studies lasting up to 8 wk with ≤6 mg Cu/d did not change indexes of copper status, if long-term copper supplementation results in the accretion of total body copper, then changes in indexes of copper status might be expected. The results of the current study show that increases in some indexes of copper

status can occur after 5 mo of a high copper intake. The significant changes that were observed in SOD, ceruloplasmin activity, and BAO activity were found under the highly controlled conditions of our metabolic research unit. These were accompanied by increased urinary copper and hair copper concentration. Although urinary and hair copper represent routes of excretion, the amounts excreted by these routes were small and the increases would be expected to have little effect on copper retention. Taken together, these findings suggest that long-term high copper intake may alter copper status. The physiologic significance of these changes is unknown.

The differences in indexes of copper status (ceruloplasmin protein and activity, plasma copper, and urinary copper) that we observed between subjects in the current study are consistent with our previous observations. The reasons for these differences are not known but presumably reflect, in part, subtle differences in genetic backgrounds, lifestyle habits, and physiologic status.

Antioxidant status

The in vivo ratio of DHA to total ascorbate has been suggested to be a biomarker of oxidant stress, and the plasma ratio of DHA to ascorbate is increased under the oxidant stress of smoking (36). We gave a large dose of vitamin C near the end of MP-A and MP-B to test the hypothesis that high copper and vitamin C intakes may promote oxidant stress in vivo by releasing free copper from its binding proteins and thereby catalyzing the generation of reactive oxygen species (15, 37). However, the present results do not support this hypothesis. Whereas plasma ascorbate doubled at 4 h postdose, the percentage of ascorbate as the oxidized DHA form was not affected by the changes in plasma ascorbate or copper intake. This may be because the copper part of the catalytic copper-ascorbate couple was not significantly different at the end of the low and high copper intake periods.

Because dietary copper overload of rats results in increased lipid peroxidation products, malondialdehyde, conjugated dienes, and TBARS in liver and lysosomal membranes (13), we measured plasma malondialdehyde and urinary TBARS at the end of the low and high copper intake periods. There was no significant effect of copper intake on plasma malondialdehyde values. This is consistent with recent findings that supplements containing 3–6 mg Cu/d added to the usual diet of healthy adults for ≥ 6 wk had no prooxidant effect as measured by the resistance of LDL and red blood cells to in vitro oxidative stress (38, 39). In the present study, urinary TBARS were modestly higher after the high-copper period than after the low-copper period. However, the mean urinary TBARS value of 52 ± 4 nmol $\cdot$ kg^{-1} $\cdot$ d^{-1} after the high copper intake is similar to mean values of 55 and 60 nmol $\cdot$ kg^{-1} $\cdot$ d^{-1} reported for 9 healthy American men (40) and 5 healthy Japanese men (41), respectively. Mean urinary TBARS values in those 2 studies increased to 84 and 89 nmol $\cdot$ kg^{-1} $\cdot$ d^{-1} with the consumption of salmon (40) and in vitro treatment of the urine with the oxidant t-butyl hydroperoxide (41). The increase in urinary TBARS during MP-B suggests that the copper supplements of MP-B may have had a small effect on oxidant stress in vivo.

Immune function

In the current study, high intakes of copper significantly reduced the percentage of circulating neutrophils, serum IL-2R, and the antibody titer against the Beijing strain of influenza. The average response to other viral strains of influenza after supplementation tended to be lower, but the difference was not significant. The number of circulating lymphocytes increased significantly, and the average inflammatory response as measured by the concentration of serum IL-6 tended to be higher during supplementation but not significantly so. Effects of high copper intake in this human study are consistent with those of copper deficiency or marginal copper intake (18–20). Because IL-2R regulates lymphocyte proliferation, and its concentration decreased with high copper intake, these results imply a reduction in lymphocyte proliferation. This is consistent with the results obtained with administration of high copper to mice (16) and the reduction in lymphocyte proliferation in older mice that naturally acquire high serum copper concentrations (17). Overall, the results from the present and our previous human study (20) support claims based on animal models that both copper deficiency and excessive intake modulate the immune response.

Future long-term studies with larger numbers of human subjects are needed to confirm these results and evaluate their physiologic significance. Some of the changes that we observed (eg, increases in SOD, ceruloplasmin activity, BAO, IL-2R, urinary TBARS, and changes in immune response) may suggest an adverse effect of high copper intake.

Summary

We showed previously that copper metabolism is highly regulated (42). With adequate and low dietary copper, indexes of copper status do not change until intake is very low (4, 22). The results of the present study provide further evidence of a high level of homeostatic control of copper metabolism. However, our results showed that a high copper intake over a period of several months changes some indexes of copper status, antioxidant status, and immune function. The physiologic implications of these effects are not yet known.

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