Copper Supplementation of Adult Men: Effects on Blood Copper Enzyme Activities and Indicators of Cardiovascular Disease Risk

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In rats, copper deficiency leads to low copper metalloenzyme activity, high serum cholesterol, and cardiovascular lesions. In humans, moderately low copper intake may be common, but the consequences remain largely uncertain. The present study examined the effects of copper supplementation (2 mg/d for 4 weeks in a copper/placebo crossover design) in 20 adult men with moderately high plasma cholesterol. End-point measurements were three copper enzyme activities, erythrocyte superoxide dismutase (SOD), plasma ceruloplasmin (Cp), and plasma diamine oxidase (DAO), and three parameters related to the risk of cardiovascular disease (CVD), plasma cholesterol, plasma lipoprotein (a) [Lp(a)], and lag times for very–low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) oxidation in vitro. Although copper had no significant effects on any parameter for the entire study group, it did significantly increase two enzyme activities (SOD and DAO), as well as lipoprotein oxidation lag times, in 10 subjects in the lower half of a median split for precopper values. Thus, copper supplementation appeared to influence some types of measurements in subjects beginning with less than median values.

IN EXPERIMENTAL ANIMALS, it is well established that copper deficiency produces low copper metalloenzyme activity and adversely affects the indices of risk for cardiovascular disease (CVD).[1-8] Examples of the latter in copper-deficient rats include hypercholesterolemia, high susceptibility of very–low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) to oxidation in vitro, abnormal arterial connective tissue, cardiac hypertrophy, and irregular electrocardiograms.[1-6] Although an extensive body of such experimental animal research exists, only a few studies have investigated the relationship between copper and CVD in humans. Two of these studies examined cholesterol values after intentionally feeding a marginal copper diet.[9,10] Two others evaluated cholesterol values after copper supplementation.[11,12] However, it is difficult to draw broad conclusions from these studies: three of them involved a combined total of only 11 subjects,[9-11] and the other study examined short-term changes in college-age males,[12] a group that may be resistant to rapid changes in plasma cholesterol.

The Food and Nutrition Board has set a safe and adequate range for copper of 1.5 to 3.0 mg/d.[13] However, several research groups have shown that the actual intake of many Americans is less than this recommendation.[14-16] This observation, plus the sheer number and variety of animal studies associating copper deficiency and cardiovascular abnormalities, justifies more inquiries on the contribution of copper intake to CVD-related parameters in humans. Therefore, this study examined the effects of 4-week copper supplementation on measurements of copper status and indices of risk for CVD. The study population was free-living men aged 31 to 52 years with baseline cholesterol values either in the higher end of the normal range or in the moderately elevated range. Such subjects were used because they might be more apt to reflect improvements in measures of CVD risk than subjects with very low cholesterol values.

Copper status was evaluated by assessing plasma ceruloplasmin (Cp) (activity and ratio of activity to immunoreactive Cp protein), and erythrocyte superoxide dismutase (SOD) and plasma diamine oxidase (DAO) activities. Cp and erythrocyte SOD activities were selected because they are the most commonly used enzymatic methods for evaluating copper status.[16] Cp activity to protein ratios were included because they can be low in copper deficiency.[17] Plasma DAO activity was included because a recent study from our laboratory has found it to be a very sensitive indicator of marginal copper deficiency in rats.[18]

The risk for CVD was assessed by measuring plasma cholesterol and lipoprotein(a)[Lp(a)] levels, and by examining the susceptibility of VLDL + LDL to lipid peroxidation in vitro. These indices were chosen for the following reasons: (1) blood tests are a minimally invasive means to perform evaluations of CVD risk in living people; (2) it is well established that elevated serum cholesterol is a risk factor for the development of CVD[19,20]; (3) an increasing body of evidence suggests that oxidized LDL promotes atherosclerosis[21,22]; and (4) Lp(a) appears to be an independent risk factor for CVD.[23]

SUBJECTS AND METHODS

Subject Selection and Protocol

Men aged 31 to 52 years with cholesterol values between 4.65 and 7.45 mmol/L participated in the study (N = 20). Subjects were recruited from the Family Practice Center at The Ohio State University. Individuals were excluded if they had a history of coronary heart disease, cerebral infarction, or diabetes. Also exempt from participation were individuals taking cholesterol-lowering medications and/or vitamin-mineral supplements containing copper. Four subjects had mild arthritis. Two admitted to a light habit of cigarette smoking (roughly one pack per week). Two were taking medication (felodipine or enalapril) to control high blood pressure. The subjects were not obese and led a sedentary to lightly active life-style. This study was approved by the Human Subjects Review Committee for Biomedical Sciences at The Ohio State University. All participants signed consent forms. Subjects were instructed to maintain their life-style patterns (ie, exercise, alcohol consumption, smoking practices, and dietary habits) throughout participation in the study.

This project used a 10-week, double-blind crossover design. For the

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first 4 weeks, subjects consumed either a copper supplement (2 mg copper as glycine-chelate; Albion Laboratories, Clearfield, UT) or a placebo (maltodextrin) supplement daily. Subjects were randomly divided into two groups by a pharmacist at The Ohio State University Hospital. After the initial 4 weeks, 2 weeks were used as a washout period in which no supplement was consumed. Then for another 4 weeks, subjects consumed the supplement not consumed for the first 4 weeks.

At baseline and 6 weeks, subjects were given a bottle containing 30 capsules and instructed to take one capsule per day for 28 days (two extra pills were provided). Subjects returned all remaining pills at 4 and 10 weeks. Blood was drawn into tubes containing EDTA at baseline and at 4, 6, and 10 weeks (15 mL per draw). Samples were collected in the morning after an overnight fast.

Assays

Plasma was isolated by centrifugation at 1,500 × *g* for 15 minutes and frozen at −20°C for most assays. Another portion of the plasma samples was used to prepare VLDL + LDL. This fraction was isolated with a precipitation procedure developed by Finley et al.[24] It was used to examine the susceptibility of VLDL + LDL to oxidation. Erythrocytes were treated as described previously[25] and stored at −20°C until assay for SOD.

Cp was determined by oxidase activity toward *p*-phenylenediamine (Sigma Chemical, St Louis, MO) as described by Rice.[26] Units were arbitrarily designated as the change in absorbance multiplied by 0.01 and expressed as units per liter. Cp protein was assessed using radial immunodiffusion plates (The Binding Site Limited, Birmingham, England) according to the manufacturer's instructions. Erythrocyte SOD activity was measured by the modified pyrogallol autoxidation assay of Prohaska[7] as adapted by DiSilvestro and Marten.[8] Plasma DAO activity was analyzed by the colorimetric assay described by Takagi et al.[27]

Cholesterol concentrations were determined using a kit purchased from Sigma. The method is based on the enzyme-catalyzed formation of hydrogen peroxide coupled with peroxidase to form a quinoneimine dye that absorbs at 500 nm. VLDL + LDL oxidation in vitro was stimulated by 8 μmol/L copper sulfate and monitored by conjugated diene formation using absorbance at 234 nm as described by Esterbauer et al.[28] The cholesterol concentration of the EDTA-free, non-HDL fraction was adjusted to 75 μg/mL for the oxidation measurements. Samples were incubated at 37°C, and absorbance readings were recorded every 10 minutes. The lag phase was determined by drawing a line tangent to the propagation phase of the curve and extrapolating this line through the horizontal axis.[29] The interval between copper ion addition and the intersection point on this axis was defined as the lag time. Lp(a) levels were measured by an enzyme-linked immunosorbent assay kit purchased from Boehringer Mannheim (Indianapolis, IN).

Statistical Analysis

Data were analyzed by the Statistical Analysis System (SAS) program (SAS Institute, Cary, NC). A paired *t* test was used to evaluate the effects of copper and placebo treatments. Significance was set at P less than .05.

RESULTS

Subjects had a mean age of 41 ± 7 years with baseline cholesterol levels of 4.65 to 7.45 mmol/L. Based on pill return, all participants showed compliance. In the total study population, copper supplementation did not significantly affect the activity of plasma DAO, erythrocyte SOD, or plasma Cp, nor the ratio of Cp activity to immunoreactive protein (Tables 1 and 2). The same was true for plasma cholesterol levels (Table 3), the susceptibility of VLDL + LDL to oxidation in vitro (Table 3), and Lp(a) levels (297 ± 30 *v* 298 ± 307 mg/L). Lp(a) levels seemed to be race-dependent: subjects of African descent (n = 4) had a mean baseline level almost three times that of caucasian subjects (n = 16).

Table 1. Copper-Supplementation Effects on Plasma CP

	Activity (U/L)	Protein (mg/L)	Activity to Protein Ratio (U/mg protein)
Preplacebo	126 ± 36	442 ± 66	0.28 ± 0.06
Postplacebo	133 ± 33	454 ± 82	0.30 ± 0.05
Precopper	131 ± 33	470 ± 106	0.29 ± 0.07
Postcopper	135 ± 27	465 ± 104	0.29 ± 0.04

NOTE. Values are the mean ± SD for 20 subjects. Results were obtained after 4 weeks of copper or placebo consumption with a 2-week washout period. No presupplement values were significantly different from the respective postsupplement values (paired Student's *t* test).

For all indices examined, there was no significant difference between precopper and preplacebo values. This suggested that 2 weeks was an adequate time for a washout period with this protocol.

Although copper supplementation did not exert significant effects when considering the entire study population, a trend was noted upon an alternative examination of the data. Copper effects on erythrocyte SOD activity, plasma DAO activity, and VLDL + LDL oxidation lag time were analyzed by a median-split approach based on precopper values. In 10 subjects with values less than the median, copper produced statistically significant increases (Table 4). In contrast, the placebo did not produce any significant increases in these same subjects.

DISCUSSION

Copper supplementation did not significantly affect copper status indices or CVD risk factors in the entire group, but did affect some parameters for subjects with precopper values less than the median. One of these parameters was the activity of the copper enzyme erythrocyte SOD. Interestingly, in a previous study by our laboratory,[25] copper-supplement effects on arthritis patients show a resemblance to the SOD results of the present study. In the former study, copper supplementation elevated SOD activity in most but not all patients. Patients who were unaffected started with SOD values at or above the mean control value (about 3,400 U/mL packed cells). Similarly, in the present study, all subjects in the lower half of the median-split had precopper SOD activity less than 3,400 U/mL. Thus, in both the

Table 2. Effects of Copper Supplementation on Plasma DAO and Erythrocyte SOD Activities

	Plasma DAO (U/L)	Erythrocyte SOD (U/mL cells × 10^{-3})
Preplacebo	62 ± 36	3.6 ± 1.4
Postplacebo	78 ± 37	3.3 ± 1.0
Precopper	62 ± 38	3.3 ± 0.8
Postcopper	75 ± 25	3.2 ± 0.8

NOTE. Values are the mean ± SD for 20 subjects treated as in Table 1. None of the presupplement values were significantly different from the respective postsupplement values (paired Student's *t* test).

Table 3. Effects of Copper Supplementation on Plasma Risk Factors for CVD

	Preplacebo	Postplacebo	Precopper	Postcopper
Cholesterol (mmol/L)	5.51 ± 0.76	5.68 ± 0.94	5.53 ± 0.68	5.61 ± 0.85
VLDL + LDL oxidation lag time (min)	61 ± 27	63 ± 32	56 ± 36	60 ± 17

NOTE. Values are the mean ± SD for 20 subjects treated as in Table 1. No presupplement values were significantly different from the respective postsupplement values (paired Student's *t* test).

Table 4. Effects of Copper Supplementation on Selected Parameters Analyzed as a Median-Split

	Plasma DAO (U/L)	Erythrocyte SOD (U/mL cells × 10^{-3})	VLDL + LDL Oxidation Lag Time (min)
Above median			
Precopper	90 ± 28	4.0 ± 0.5	75 ± 40
Postcopper	81 ± 20	3.3 ± 0.7	56 ± 17
Below median			
Precopper	34 ± 21	2.6 ± 0.5	36 ± 6
Postcopper	68 ± 27*	3.0 ± 0.7†	64 ± 15*

NOTE. Median-splits were based on precopper values. Values are the mean ± SD for 10 subjects.

*$P < .01$ (paired Student's *t* test).

†$P = .05$ (paired Student's *t* test).

former and present studies, the copper-supplement effects on SOD activity depended on starting values.

Plasma DAO activity, like erythrocyte SOD activity, was increased by copper supplementation in subjects with less than median precopper values. Both SOD and DAO activities can differentiate between marginal and adequate copper status in rats.[8,18] In humans, erythrocyte SOD has shown mixed results for apparently marginal copper status. For example, Turnlund et al[30] found that erythrocyte SOD activity did not significantly decline in 11 young men after a 42-day feeding of a low-copper formula diet. In contrast, using a different low-copper feeding protocol in an older group of men, Reiser et al[31] did find a decrease in erythrocyte SOD activity. DAO has not yet been tested extensively with regard to human copper status. However, DAO was found to be very low in a human subject with severe copper deficiency.[18]

The present study also assessed the VLDL + LDL oxidation lag time, a parameter believed to be related to atherosclerosis.[21,22] Lipoprotein lag times can be affected by many factors, and humans exhibit high variability.[32] In our study, one lag time was 180 minutes before copper consumption, and another lag time was 180 minutes after placebo consumption. These extreme outliers skewed the mean lag time to the right. This high degree of variation justifies the use of an index somewhat resistant to outlying values, such as the median. In the present study, VLDL + LDL oxidation lag times were altered by copper in subjects with less than median precopper values. This result, combined with the observation that copper-deficient rats show low lipoprotein oxidation lag times,[4] justifies further study of human copper status and lipoprotein oxidation.

Although there were no significant effects of copper on any tested parameter in the total study population, an effect may have been seen with a longer supplementation period or different subject types (ie, the elderly or people with health problems). This supposition could apply both to parameters that were affected in subjects below the median-splits and to parameters not affected at all. In support of the latter supposition, there is a preliminary report that 8 weeks of copper supplementation can decrease plasma cholesterol in a small number of adult males.[11]

One noteworthy negative result in the present study is the lack of support for the speculation that high copper intake can contribute to lipoprotein oxidation in vivo. Copper supplementation showed no signs of reducing the lag time for VLDL + LDL oxidation. If high copper intake did reduce lag time, then this could be proposed to lead to atherosclerosis, a condition that may be fostered by lipoprotein oxidation.[21,22] In contrast, two studies[33,34] suggest that poor copper status may contribute to atherosclerosis. In one study,[33] subjects with this condition show low mononuclear cell copper. In the other study,[34] subjects with atherosclerosis show low aortic Cu-Zn SOD activity.

Our Lp(a) data resemble previous findings showing Lp(a) values to be race-dependent, nonresponsive to dietary changes, and highly variable between individuals.[35-37]

In conclusion, copper supplementation did not significantly alter the parameters under consideration for the whole study population. However, median-split analysis justified further inquiry into the concept that many individuals would benefit from increased copper intake.

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