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**Marco A. Méndez, Magdalena Araya, Manuel Olivares,
Fernando Pizarro, Mauricio González**

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Sex and ceruloplasmin modulate the response to copper exposure in healthy individuals.

Marco A. Méndez, Magdalena Araya, Manuel Olivares, Fernando Pizarro, Mauricio González.

Institute of Nutrition and Food Technology, University of Chile.

Mailing address:
Marco A. Méndez PhD
Institute of Nutrition and Food Technology (INTA)
University of Chile
Macul 5540
Santiago 11 - CHILE
Phone: 56 (2) 678 1545
Fax: 56 (2) 2214030
Email: mmendez@inta.cl

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List of abbreviations: Cp= Ceruloplasmin; GOT= glutamic-oxalacetic transaminase); GPT =glutamic-pyruvic transaminase); GGT gamma glutamyl transferase; DA =Discriminant Análisis; DMPS¹=Dimercapto-1-propansulfonsäure(DMPS), natrium salt, monohydrate; DMPS (0-4)= urinary copper excretion from 0-4 hours after administering 300 mg; DMPS (5-24)= urinary copper excretion from 5-24 hours after administering 300 mg DMPS (24h)= 24 hour urinary copper excretion after administering 300 mg DMPS; MNC= mononuclear cells; PCA= Principal Component Analysis; LDA= Linear Discriminant Analysis; TDI= Tolerable daily intake.

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Abstract

Previous studies indicated that sex might influence the response to copper exposure. Ceruloplasmin is an indicator of copper status but it is not clear whether and how it reflects changes of copper status among healthy population. In this study, eighty-two apparently healthy women and men were chosen among 800 individuals because their ceruloplasmin values belonged to the higher and lower 10% in the group ceruloplasmin distribution curve, respectively. Before and after receiving a supplement of 10 mg Cu/day (Upper Limit of Daily Intake), for two months, blood and urinary biochemical measurement of potential copper markers were performed. Principal Component Analysis and Linear Discriminant Analysis were used to identify blood and/or urinary copper indicators that showed a differential response to copper. Results showed that Cp values in serum represent a reliable indicator to differentiate subgroups within the normal population in their response to copper exposure. The response depends on Cp values and on sex, such that women with higher and men with lower Cp values exhibit the greatest response.

Copper is required for the function of several cuproenzymes, therefore its presence is essential for different physiological functions (Linder 1991). Copper, however, is able to generate free radicals and oxidize cellular component through its redox activity (Aust et al. 1985). These conflicting properties demand a close regulation of the metal at the organism level. Effects associated with severe lack or excess copper are well described in genetic conditions, such as Menkes (Chelly et al. 1993; Mercer et al. 1993; Parano et al. 1996; Vulpe et al. 1993) and Wilson (Bull et al. 1993; Tanzi et al. 1996) diseases. In contrast, much less is known about relevant biological effects associated with copper deficiency and excess when no mutation is present (Davis 2003; Hambidge 2003). It is well known that serum ceruloplasmin and copper values are higher in young children and increase during even mild inflammatory/infectious processes, but their relations to copper intake and markers of copper status are not clear and data available suggest that they modify only when exposure changes by several orders of magnitude (Araya et al. 2003a; 2003b). Whether marginal or moderate changes in copper exposure may result in adverse effects to human health is still not clear, because there are no sensitive indicators of marginal changes in copper status and because early functionally relevant responses are not well defined.

With the aim of improving our understanding about the early effects of copper on human health we have conducted a series of studies on asymptomatic adults undergoing controlled copper exposure. This varied between approximately 3 to 10 times the customary dietary intake. Thus, clinical trials showed that nausea is the earliest and most frequent response and generated data to calculate the dose-response curve to acute copper exposure (Pizarro et al. 2001; Araya et al. 2003a; 2003b; 2003c; Olivares et al. 2001). A

community survey in which participants ingested between 0.9 and 10 mg Cu/day for two months (the concentration defined as Tolerable Daily Intake (TDI) of copper ingestion for humans (Araya et al. 2003b)) allowed describing the full range of responses to copper exposure, showing that there are more gastrointestinal responses (mainly nausea) at increasing copper exposure (copper concentration in water) and that these responses diminish with time, suggesting adaptation (Araya et al. 2004). In all these studies the statistical analyses suggested that the variable sex influenced the results.

No changes in biochemical blood parameters were detected in a previous study in which healthy participants were exposed to up to 6 mg Cu/L of water, which represented as much as 14 mg Cu/D on occasional days depending on the volume of fluids ingested (Araya et al. 2003b). To further assess the homeostatic responses to copper exposure in a recent study we assessed the effects of exposing asymptomatic adults grouped by their ceruloplasmin values to the tolerable daily intake (TDI) for two months, administered as a single daily supplement. A series of biochemical responses of blood and urinary potential copper indicators were measured and their detailed analysis will be reported elsewhere (Araya et al. submitted). Considering the insufficient evidence to decide on the appropriateness of the different indicators proposed to assess copper status among apparently normal individuals, in this paper we present a multivariate strategy that evaluated a series of proposed indicators for their capacity to identify differences within the apparently healthy group. We performed our analysis as a function of sex, including the relative importance of each measurement before and after a copper supplementation period.

Material and Methods

The study was a prospective controlled trial in healthy adults. Participants were 18-50 years and approximately 50% were over/below 30 years. One half of them were women, who were not pregnant and did not get pregnant throughout the study. The need for volunteers was advertised in the southeastern area of Santiago; potential participants received detailed information about the protocol and those who accepted to participate signed an informed consent prior to forming the study groups. The Committee on Ethics for Human Research, INTA, University of Chile approved the protocol. All individuals received 10 mg Cu/d administered under direct supervision as 2 gelatin-coated 5 mg copper tablets (as copper sulfate). This dose was chosen based on two criteria: Data published by Pratt et al (1985) showed no significant changes in hematocrit, triglyceride, SGOT, GGT, LDH, cholesterol, or alkaline phosphatase in adults (human beings) after administering 10 mg of copper/day as copper gluconate or placebo capsules for 12 wk. Secondly, Upper Limit (ULs) (10 mg Cu/d) is defined as the maximum intake from food, water and supplements that is unlikely to pose risk of adverse health effects from excess in almost all (97.5%) apparently healthy individuals, in an age- and sex-specific population group (Institute of Medicine, Food and Nutrition Board, 2001). We hypothesized that individuals may have a differential response to copper supplementation depending on their position in the serum ceruloplasmin distribution curve, considering a lower value as an index of long-term low intake. Dietary surveys assessing total daily copper intake from food and water in Chile has revealed that 16.4% of men and 33.3% of women between 20 and 60 years are below the estimated average requirements (EAR) (Olivares et al 2004). Accordingly, 800 apparently healthy individuals were screened for their serum ceruloplasmin protein, and 82

individuals that represented the 10% higher (C_{high} group) and 10% lower (C_{low} group) values in the C_p distribution curve were assessed (n= 41 for each group). Inclusion criteria were being free of acute infectious/inflammatory processes (C-reactive protein below 0.8, as indicated by the kit manufacturer and white cell count in hemogram below 12.000 cells/ml, lower limit of the normal range (Dalman et al. 1977), of chronic illnesses and of chronic multi medication that may interfere with the study.

Before and after the two-month copper supplementation blood and urine studies were carried out. These studies included 18 potential markers of copper status chosen from published data and our experience. Studies in blood included measurement of copper by atomic absorption spectrometry, in serum (Perkin Elmer Model 2280, Norwalk, Conn.) and in peripheral mononuclear cells (MNC) (Perkin Elmer, Model SIMAA 6100, The Perkin-Elmer Corporation, Norwalk,CT, USA), C_p protein measured by nephelometry (array protein system; Beckman Instruments Inc Brea, CA), liver enzymes (sGOT, sGPT and GGT) determined by commercial kit (Boehringer Mannheim, Mannheim, Germany), homocysteine values determined by an Abbott Kit (IMX system homocysteine Abbott Laboratories, Diagnostic Division, Abbott Park, IL), Zn-Cu-superoxide dismutase activity in erythrocytes measured with a commercial kit (Bioxytech SOD-525 Assay, OXIS International Inc, Portland OR) and glutathion measured in peripheral mononuclear cells (PMNC) using a Glutathione Assay Kit (Calbiochem). Studies in urine included measurement of urinary copper excretion after a chelator challenge with 300 mg 2,3-dimercaptopropane-1-sulfonate (Dimaval ®, Heyl Laboratory, Berlin, Germany), within 3 days before and after supplementation beginning and end. Calculating sample size was

using α at 5% and power at 80% 35-45 individuals per group were needed to detect a delta of 0.5 SD in the biochemical measurements that were planned.

Data were analyzed using SYSTAT 5.0 (SYSTAT, Inc., Evanston, Illinois; Wilkinson 1996). All data were log transformed in order to meet the assumptions of normality of data. Because study groups were formed on the basis of individuals' serum Cp values (Cphigh and Cplow) data were first assessed by univariate analysis at the beginning and end of the supplementation period to determine whether groups formed by individuals' Cp values were significantly different. The integral (multivariate) response to copper exposure (defined as the response of all biochemical measurements at the same time) was explored by multivariate analysis, using PCA and LDA. PCA is a multivariate statistical tool that simplifies complex data sets transforming the original variables into new independent and uncorrelated variables named principal components, which explain the observed variability in decreasing order. Thus, the first components concentrate maximal information (variance explained) about the analysis; additionally, for each component there is an eigenvalue with an associated variance value (explained variance). On the other hand, we used LDA as a classification function to calculate scores for each variable in the different groups; LDA permits evaluating whether there are significant relationships between qualitative variables or classes (in this case, sex and lowCp and highCp groups) and quantitative predictor variables (in our case, eigenvalues of each biochemical variable). Since we knew the classes we built a linear discriminant function to estimate the goodness of this classification within each class. A matrix originated by PCA served as the basis for LDA input data, in all analyses we added eigenfactors until obtaining close to 80 % of variability in the model. Thus, the LDA output allowed assessing 1) whether sex and Cp

levels (lowCp and highCp) differences are associated with responses to copper, and 2) the integral (multivariate) response of individuals to copper exposure. Additionally, because the discriminant function was applied to the same sample used to derive it, we used both cross-validation and jackknife procedures to obtain unbiased estimates (Hair et al 1992). Using these procedures we obtained a classification matrix that allowed evaluating the performance of the defined classes (sex and lowCp and highCp groups), verifying which individuals had high values of correct classifications. As a control, we also assessed whether the analyses performed using both cross-validation and jackknife procedures yielded the same values of correct classifications; since no difference were found the results are presented only following the output of the jackknife matrix. All the LDA data showed normal multivariate distribution, which was evaluated using Sen and Puri test (Sen and Puri 1968).

Results

Univariate analysis. As expected, Cp values of individuals grouped by Cp group (Cplow and Cphigh) and sex showed significant differences at beginning (ANOVA: Cp group :F= 103.99 , d.f.= 1 , p < 0.0001 ; Sex: F= 22.256 , d.f= 1, p< 0.0001; Interaction Cp group*Sex F= 10.591, d.f.= 80, p < 0.002; Fig. 1), and Final of treatment (ANOVA: Cp group: F= 126.710, d.f.= 1 , p < 0.0001, Sex: F= 22.256 , d.f= 1, p< 0.0001; Interaction Cp group*Sex F= 10.591, d.f.= 80, p < 0.008; Fig. 1).

Multivariate analysis. As a first step we explored whether differences existed when all biochemical measurements were considered at the same time (integral response), assorting individuals both by sex and Cp groups. PCA showed that the first four components

explained 68.62 % of the variability; in the first component ferritin, GGT and GPT obtained the highest loading value (Table 1) while serum zinc, copper in serum and copper in MNC obtained the lowest values. LDA using the matrix obtained from the first six PCA components (which explained over 80.8% of the variability observed) and using sex as classification variable revealed statistically significant differences between sexes (Wilks' lambda= 0.423; F= 17.043; d.f.= 6,75; p< 0.0001). Discriminant classification matrix showed high values of correct classifications (80% for men and 88% for women), indicating that there were differences related to sex associated with the parameters evaluated. In view of these results the next analyses were performed separately on women and men, before and after copper supplementation.

Before copper supplementation. In women, PCA showed that the first four components explained 70.6 % of the variability. In the first component, ferritin, GGT and GPT obtained the highest loading value (Table 2). Among men, PCA showed that the first four components explained 70.38 % of the variability. Comparing to women, the relative importance of each element in the first component somewhat differed, GPT and GGT were included, but not ferritin, and Dimaval (1-4 hrs) was among the variables with the highest loading value (Table 2). The LDA analysis performed using high and low Cp group as classificatory variables revealed that these groups were statistically different (Wilks' lambda= 0.5136; F= 5.3673; d.f.= 6,34; p< 0.0005), showing high values of correct classifications in the classification matrix (Cplow group: 80% and Cphigh group: 81%). This analysis also shows that although differences between the Cp group were significant (Wilks' lambda= 0.660; F= 2.919; d.f.= 6,34; p< 0.020) the values of correct classifications were lower than in women (Cplow group: 70% and Cphigh group: 67%).

After copper supplementation In women, PCA showed that the first four components explained 73.27 % of the variability. In the first component, as before copper supplementation, GGT, ferritin, and GPT obtained the highest loading value (Table 2). The LDA showed significant differences between the Cp groups (Wilks' lambda= 0.574; F= 4.074; d.f.= 6, 33; p< 0.0003), and a classification matrix with values of correct classifications over 50 % (Cplow group= 76 % and Cphigh group= 68%). Among men, PCA showed that the first components explained 65.91 % of the variability. In the first component GGT, GPT, and GOT showed the highest loading value, showing that in comparison with analysis before copper supplementation, GOT replaced Dimaval (1-4 hrs) (Table 2). LDA in men also showed differences between Cp groups (Wilks' lambda= 0.584; F= 2.496; d.f.= 8,32; p< 0.016), and as in the case of women, values of correct classifications were over 50 % (Cplow group= 60% and Cphigh group= 67%). Both in women and men the percentages of correct classification were lower in comparison to the figures obtained before copper supplementation.

Discussion

It is well known that serum ceruloplasmin and copper vary responding to rather minor inflammatory and infectious events; at the same time, these indicators are used to assess changes in copper status in pathological situations. To what extent they may reflect mild yet relevant changes of copper status among apparently healthy individuals is still a matter of debate (Kehoe et al. 2000; Hambidge 2003; Davis 2003; Araya et al. 2003a; 2003b). In this study participants were healthy and remained clinically healthy during the two-month controlled copper exposure. Serum aminotranferases activities are the traditional

biochemical blood measurements used on clinical ground to assess liver function. Participants received a daily copper dose defined as the upper safe limit for human consumption, therefore toxic responses were not expected, liver aminotranferases were evaluated to satisfy ethical considerations. There were no responses detected that may represent toxic effects of the copper dose used.

Both the univariate and bivariate analyses support the idea that Cp values in serum represented a reliable indicator of copper status responding to chronic copper exposure and that sex was indeed a factor that modulated the response. It is interesting that both in women and men and prior to and after copper supplementation, GGT and GPT were always included in the first component with high loading values; ferritin instead, was included only among women, while urinary copper excretion after DMPS challenge was included among men; it is difficult to interpret these differences with the present data, there is little experience with DMPS challenge among normal population; these findings deserve further research. The LDA also showed, both in women and men, that the biochemical indicators measured were significantly associated with the Cp values, the classification matrix showed that the correct assignment values were higher among women (about 80%) while among men they reached valued of about 70%, suggesting that the Cp value is a good indicator to separate the groups, a reliable descriptor of the integral response to copper exposure, but it may be more sensitive for women that for men.

By the end of the copper exposure period, in both sexes the first four PCA components explained a lower proportion of variance (73.3% in women and 65.9% in men), suggesting that variability increased in the groups after copper supplementation. It is intriguing that among women ferritin decreased its loading value while GGT became the most relevant variable (Table 2). Among men GGT is also the main variable explaining the

variance, and the three transaminases as a whole (GGT, GPT and GOT) are the factors with the highest loading values. This is a relevant finding because these enzymes classically change in hepatic diseases, but it is not clear that they respond to subclinical situations (Jones et al. 1997; Cashman et al. 2001; Lowe et al. 2002; Olusi et al. 2003; Nayak et al. 2003); even accepting that they may respond to other illnesses, our results support their use to monitor potential adverse effects of copper in the liver.

Conclusion

We conclude that Cp values in serum represents a reliable indicator of copper status and of the host response to copper exposure. This information is relevant to risk assessment studies of copper effects in human health and environmental epidemiology. This response depends on sex and also on the Cp value, such that women with higher and men with lower Cp values exhibit the greatest response. Why women respond different from men and why apparently healthy individuals respond differently depending on their Cp values is not clear, ongoing studies are currently exploring these aspects.

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TABLE 1.

Values (loadings) in the first four principal component axes (PCA) for each of the 18 variables assessed in adult individuals before copper supplementation.

VARIABLES	PC1	PC2	PC3	PC4
Hemoglobin	0.104	0.049	-0.184	-0.018
Ferritin	1.347	0.077	-2.606	1.396
Homocysteine	0.127	-0.104	-0.177	-0.719
Serum Cu	-0.003	0.418	0.917	-0.300
Serum Fe	0.169	0.578	-0.365	-0.397
Serum Zn	-0.003	0.002	-0.086	0.247
Cu in MNC	-0.005	-0.066	0.130	0.075
Fe in MNC	-0.013	-0.143	0.128	-0.294
Zn in MNC	0.017	-0.099	-0.023	-0.105
GOT	0.542	-0.437	1.328	0.929
GPT	0.945	-1.187	1.111	1.372
GGT	1.680	0.659	1.342	-1.571
SOD	0.223	0.264	0.395	1.491
DMPS1	0.261	2.124	-0.240	0.025
DMPS2 (4 h)	-0.333	0.502	0.782	2.621
DMPS3 (5-24 h)	-0.052	0.830	0.619	-0.217
DMPS4 (0-24 h)	-0.318	1.199	0.443	1.185
Urinary Cu	0.058	0.648	0.038	0.297
% variance explained	29.08	18.11	12.8	9.650

List of abbreviations: GOT= glutamic-oxalacetic transaminase); GPT =glutamic-pyruvic transaminase); GGT gamma glutamyl transferase; DMPS¹=2,3-dimercaptopropane-1-sulfonate; DMPS²= urinary copper excretion from 0 to 4 hours after administering 300 mg DMPS; DMPS³= urinary copper excretion from 5-24 hours after administering 300 mg DMPS; DMPS⁴= urinary copper excretion from 0-4 hours after administering 300 mg DMPS; MNC= mononuclear cells.

TABLE 2

Loadings from the first principal component axes (PCA) studied before and after copper supplementation. In bold are represented those variables with highest loading in the first component.

VARIABLES	BEFORE		VARIABLES	AFTER	
	WOMEN	MEN		WOMEN	MEN
Hemoglobin	0.059	0.006	Hemoglobin	0.021	-0.029
Ferritin	1.161	-0.346	Ferritin	1.553	0.428
Homocysteine	-0.034	-0.147	Homocysteine	0.147	0.293
Serum Cu	0.263	0.038	Serum Cu	0.295	0.132
Serum Fe	0.132	0.093	Serum Fe	0.130	-0.106
Serum Zn	0.006	0.058	Serum Zn	-0.033	-0.125
Cu in MNC	0.001	-0.074	Cu in MNC	0.116	0.090
Fe in MNC	-0.005	-0.112	Fe in MNC	0.076	0.102
Zn in MNC	0.018	-0.094	Zn in MNC	0.072	0.143
GOT	0.679	-0.993	GOT	0.722	0.870
GPT	0.848	-1.640	GPT	0.781	0.885
GGT	1.656	-1.197	GGT	2.175	2.540
SOD	0.326	0.012	SOD	-0.152	-0.321
DMPS1	0.716	0.908	DMPS	0.512	0.844
DMPS2 (4 h)	0.165	0.675	DMPS4 (0-24 h)	0.472	-0.441
DMPS3 (5-24 h)	0.104	0.206	Urinary Cu	0.497	0.337
DMPS4 (0-24 h)	-0.229	0.512			
Urinary Cu	0.100	0.279			
% variance explained	31.58	29.58		29.57	26.19

List of abbreviations: GOT= glutamic-oxalacetic transaminase); GPT =glutamic-pyruvic transaminase); GGT gamma glutamyl transferase; DMPS¹=2,3-dimercaptopropane-1-sulfonate; DMPS²= urinary copper excretion from 0 to 4 hours after administering 300 mg DMPS; DMPS³= urinary copper excretion from 5-24 hours after administering 300 mg DMPS; DMPS⁴= urinary copper excretion from 0-4 hours after administering 300 mg DMPS; MNC= mononuclear cells.

Figure legends.

Figure 1.- Ceruloplasmin concentration (media \pm error standard) in men and women before and after copper supplementation, in groups formed by individuals' ceruloplasmin concentrations (high= C_{high} and low = C_{low}).

FIGURE 1

