Biol Trace Elem Res (2008) 121:9–15
DOI 10.1007/s12011-007-8029-z

Copper and Liver Function Indicators Vary Depending on the Female Hormonal Cycle and Serum Hormone Binding Globulin (SHBG) Concentration in Healthy Women

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Received: 23 May 2007 / Accepted: 6 July 2007 /
Published online: 9 October 2007

Abstract Previous studies showed that responses to chronic administration of copper were significantly associated with gender, raising the need to better characterize the relation between the effects observed and stradiols. The objective of this study was to measure copper and liver function indicators and the sex hormone binding globulin (SHBG) serum concentrations in healthy adults exposed to copper, grouped by sex and phase of the female hormonal cycle. Healthy females on day 7 (follicular phase, Group 1, $n=39$), on day 21 (secretory phase, Group 2, $n=34$) and males (comparison group, Group 3, $n=34$) received 8 mg Cu/day (as copper sulfate), orally, for 6 months. On days 0, 30, 60, 120, and 180, the serum concentration of copper, ceruloplasmin, liver aminotransferases, and SHBG were measured. Analysis of results included analysis of variance (ANOVA; repeated measures) and the post hoc Bonferroni correction. Participants remained healthy throughout the study period, including aminotransferases below the cut off in all measures. GGT, AST, and ALT activities were significantly different by group and by time (ANOVA repeated measures $P<0.05$). Six-month curves of serum copper and ceruloplasmin concentrations were different by group, by time and interaction group×time (all $P<0.001$). SHBG curves were different by group and time ($P<0.01$), and interaction group×time ($P<0.009$). Serum copper, ceruloplasmin, and liver aminotranferases are influenced by estrogens/progesterone, something that should be considered when these indicators are used as outcomes of effects. Time of sampling was also significantly associated with the indicators and deserves further study.

Keywords Copper · Men · Women · SHBG · Ceruloplasmin · Liver aminotransferases

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Introduction

Early effects of chronic copper exposure are not well defined, making it difficult to differentiate effects, which may represent potential risks for human health from adaptive changes that have no relevant functional consequences [1, 2]. In recent years, several studies evaluated the responses to controlled copper exposure using copper doses that are in the high range of the Upper Limit (UL), i.e., the amounts of daily copper intake defined as safe for human consumption [3, 4]; in these studies, indicators of copper status, liver function, oxidative stress, and others measured are outcomes of effects [5–9]. In a previous study, a cohort of healthy women and men were searched for effects after copper dosing of 10 mg Cu/day, administered for 2 months. Using different statistical approaches for data analyses, we found that both the acute and chronic responses detected differed significantly by sex [6, 10].

These data raise the need to better characterize the differences in responses by sex, demonstrating whether the association is with stradiols. One way to approach this is by assessing the effects of copper dosing in women that are evaluated in the different phases of their hormonal cycle: the follicular phase (characterized by comparatively lower progesterone plasmatic concentrations) and the secretory phase (with higher levels of this hormone). The sex hormone binding globulin (SHBG) is a relevant protein involved in the transport and metabolism of sex hormones. Produced in the liver, three isoforms have been described; structural studies of the protein describe three binding sites: site I binds Ca^{+2}, site II binds divalent metals (including Cu^{+2}) and is located close the steroids binding site, and site III binds Zn^{+2} [11]. SHBG binds 90 to 99% of the steroid hormones with high affinity, leaving the unbound fraction responsible for the biological effects observed in target organs [12]. Blood SHBG concentration has been shown to increase when estrogens are administered to men and decrease when androgens are administered to women [13].

As concentrations and changes of serum SHBG correlate well with changes associated with stradiols, in this study, we assessed whether (1) the changes observed during copper dosing that appear associated with sex can be differentiated by the phase of the hormonal cycle and (2) they are associated with SHBG. Thus, the objective was set at determining the serum concentrations of SHBG, of copper and ceruloplasmin, and of liver enzymes activities during the follicular and secretory phases of the female hormonal cycle, during 6 months of controlled, chronic exposure to copper.

Materials and Methods

This experimental clinical assay assessed monthly a cohort of healthy women and men during 6 months, divided into three groups: women on day 7±2 of their hormonal cycle (Group 1, n=39), women on day 21±2 of their hormonal cycle (Group 2, n=34), and a group of men that acted as comparison group (Group 3, n=34). Each day, participants ingested one gelatin capsule containing 8 mg of copper (as copper sulfate), under directed supervision. Copper dosing was chosen on the basis of previous studies that showed that customary copper intake in the population group that originated the study group is in the low range of the adequate oral intake [14] and that this dosing does not induce adverse effects (unpublished). Candidates to the study were called by local advertising and fulfilled the following inclusion criteria: They were free of symptoms, had no known pathological conditions, and their blood biochemistry was within normal values, including serum copper and ceruloplasmin, glycemia, liver aminotransferases hemoglobin, and C reactive protein

below the cut offs [15] (http://www.nlm.nih.gov/medlineplus/ency/article/003458.htm, accessed 052207). Potential volunteers to form women cohorts were followed for 3-months before the beginning of the protocol, and cases were selected among those who had more regular menstrual periods. Selected participants were not on oral contraceptives; 37 of 39 and 33 of 34 carried a copper intrauterine device in groups 1 and 2, respectively (Fisher *t* test, NS). Day of sampling was estimated on the basis of the last menstrual date; on each occasion, the study variables (see below) and estrogens and progesterone were measured. After receiving detailed explanations about the protocol, those who agreed to participate signed a written consent and were incorporated to the study. The protocol was cleared by the Institute of Nutrition and Food Technology (INTA) Institutional Review Board, University of Chile.

Procedures

Determinations to assess inclusion criteria prior to incorporation to the protocol included: hemoglobin and leukocytes count (Electronic Counter, CELL-DYN 1700, ABBOTT Diagnostics, Abbott Park, IL); glycemia (by a commercial kit QCA, Química Clínica Aplicada S.A., Amposta, Spain) and high sensitivity CRP (Turbox, Orion Diagnostica, Espoo, Finland). Then, on days 0, 30, 60 120 and 180 serum ceruloplasmin (protein) was measured by nephelometry (Array Protein System, Beckman Instruments Inc., Brea, CA); serum copper concentration by AAS (Perkin Elmer, Model SIMAA 6100, The Perkin-Elmer Corporation, Norwalk, CT, USA); liver aminotransferases (aspartate-aminotranferase [AAT], alanine-aminotransferase [ALAT] and L-γ-glutamyltransferase [GGT]) by a commercial kit (Química Clínica Aplicada S.A., Amposta, Spain). Cut offs amino-transferases were based on those used to diagnose liver dysfunction (35, 35 and 38 for AAT, ALAT and GGT, respectively (15, 16); SHBG by a commercial enzyme immuno assay (DELFIA®, Perkin-Elmer USA), and progesterone and estrogen by a chemiluminescence assay (Elecsys, Roche, Graz, Austria)

Statistics

Sample size was calculated to detect significant differences with deltas of 1SD in the SHBG values, with power 80%, $\alpha = 0.05$ y $\beta = 0.1$. This gave 38 individuals per group; considering a 10% drop out 42 individuals were initially incorporated to the groups. Analysis of results was planned to consider two variables: "group" and by "time of measurement" (hereon called time) ANOVA, repeated measures. Post-hoc analysis included Bonferroni correction and Pearson correlations. Statistical tests were conducted using SYSTAT 5.0 (SYSTAT, Inc., Evanston, IL, USA) [20].

Results

The protocol was completed in 107/124 individuals (86.3%). Causes for withdrawal were moving to another job ($n=3$), not following the protocol as requested ($n=4$), refusal to continue participating ($n=6$), and getting pregnant ($n=3$), without differences between the three study groups. Participants remained healthy during the 6-month study period. Mean± SEM of serum progesterone/estrogens concentrations were 1.10±0.31/ 64.9±11 in Group 1 (women in follicular phase), 7.43±1.19/118.9±13,4 in Group 2 (women in secretory phase), and 0.6±0.03/21.4±0.7 in Group 3 (men, comparison group). As expected, values

Table 1 Hemoglobin, Leukocytes Count, Glycemia, and Ceruloplasmin Concentration (Median±SEM) in Group 1 (Women, Follicular Phase), Group 2 (Women, Secretory Phase), and Group 3 (Men, Comparison Group) at Incorporation to the Protocol

	Hb (g/dl)	Leukocytes (n/mm^3)	Glycemia (mg/dl)	Ceruloplasmin (mg/dl)
Before copper dosing				
Group 1	15±0.1	7591±292	90±1.2	28±1.1
Group 2	14±0.1	7079±234	85±1.3	30±0.7
Group 3	14±0.1	7768±453	87±1.3	29±0.9

of initial glycemia, leukocytes count, hemoglobin, and serum copper and ceruloplasmin concentrations were within normal limits, and comparisons with values obtained during the 6-month study period revealed no significant differences (Table 1). Aminotransferase activities were below the cut offs in all measurements (Table 2, differences NS). The 6-month curves of GGT, AAT, and ALAT activities were significantly different by group and by time (ANOVA repeated measures $P<0.05$, Table 2), while differences of ALAT were also significant for interaction group×time (ANOVA repeated measures, $P<0.001$). Serum copper and ceruloplasmin in the 6-month curves were different by group, by time and interaction group×time (all $P<0.001$, Fig. 1). Serum SHBG curves were different by group and time ($P<0.01$) and interaction group×time ($P<0.009$, ANOVA repeated measures, Fig. 2). Post-hoc analysis with Bonferroni correction allowed the analysis of data independent of time; as expected, it showed that differences between groups 1 and 3 were significant for estrogens and progesterone concentrations ($P<0.01$); differences between groups 2 and 3 (women) were significant for GGT activity and for estrogens and progesterone concentrations ($P<0.05$). Pearson correlations between SHBG and the indicators measured were significant for the three liver aminotranferases but not significant or copper and ceruloplasmin (Table 3).

Table 2 Serum Aminotranferases Activities [L-γ-glutamyltransferase (GGT), Aspartate-aminotransferase (AAT), and Alanine Aminotransferase (ALAT)] during Copper Dosing (8 mg Cu/day as Copper Sulfate) in Group 1 (Women, Follicular Phase), Group 2 (Women, Secretory Phase), and Group 3 (Men, Comparison Group)

	Time of copper dosing (in months)				
	0	1	2	4	6
GGT (UI)					
Group 1	13±1.5	15±1.6	13±1.7	15±1.5	11±1.6
Group 2	10±1.6	13±2.0	13±2.3	14±2.3	9±2.0
Group 3	9±1.7	9±1.0	6±1.4	6±0.7	5±1.0
AAT (UI)					
Group 1	22±0.9	24±1.6	24±1.1	21±1.0	18±0.9
Group 2	20±0.9	20±0.7	20±0.8	21±1.6	18±1.1
Group 3	19±0.8	21±0.9	19±0.8	18±0.9	16±0.9
ALAT (UI)					
Group 1	23±1.5	26±2.5	26±1.5	24±1.6	22±1.9
Group 2	21±1.4	20±1.1	21±1.1	23±2.6	23±3.5
Group 3	20±0.9	20±0.8	20±1.0	19±1.0	17±1.0

Results are expressed as median±SEM. Differences by group and by time: $P<0.05$ for all three enzymes (GGT, AAT, and ALAT); interaction: $p<0.001$ for ALAT (ANOVA repeated measures).

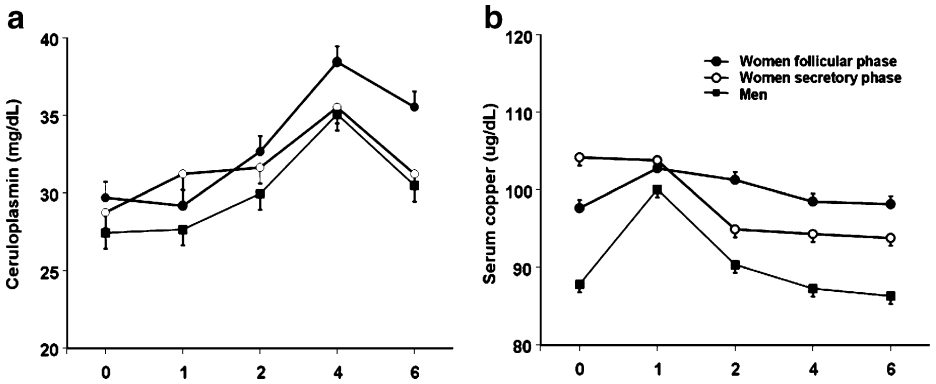

Fig. 1 Serum ceruloplasmin (**a**) and copper concentrations (**b**) during the 6-month copper dosing period (with 8 mg Cu/day, as copper sulfate) in group 1 (women, follicular phase), group 2 (women, secretory phase), and group 3 (men, comparison group). Results are expressed as median±SEM. In both curve differences by group, by time, and interaction group×time=$P<0.001$ (ANOVA repeated measures)

Discussion

Results show significant variations on serum copper concentration, ceruloplasmin, SHBG, and liver aminotransferases activities depending on sex and—in some cases—also on the phase of the female hormonal cycle. These findings are relevant to the research that searches for and intends to characterize the effects of copper in humans. Serum copper and ceruloplasmin are the most frequently used indicators of copper status, and liver aminotransferase activities represent the gold standard to assess adverse effects on liver function. Therefore, interpreting significant, yet milder changes of these indicators should take into account the variations given by sex (women and men present in the study) and, in the case of GGT, also the phase of the hormonal cycle of women participating in the studies, a variable usually not controlled.

The most difficult task in conducting this study was to warrant that the moment of sampling, representing the follicular and secretory phases of the hormonal cycle, fulfilled

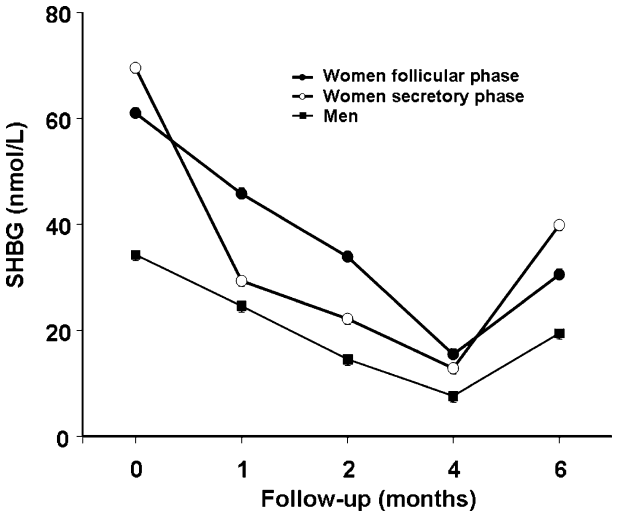

Fig. 2 Serum SHBG concentrations during the 6-month copper dosing period (with 8 mg /day as copper sulfate) in group 1 (women, follicular phase), group 2 (women, secretory phase), and group 3 (men, comparison group). Results are expressed as median±SEM. In the three groups, differences by group and by time=$P<0.001$; differences by interaction group×time=$P<0.009$ (ANOVA repeated measures)

Table 3 Correlations Between SHBG and the Copper Status, Liver Function Indicators, and Estrogens and Progesterone (Pearson Correlation Coefficient)

Variables	Pearson	*P* value
Ceruloplasmin	−0.116	0.233
Copper	−0.069	0.483
GGT	−0.322	0.001
AAT	−0.388	0,000
ALAT	−0.465	0.000
Estrogens	0.228	0.018
Progesterone	0.431	0.000

the requisites of design. The values obtained and the differences observed between the three study groups (see "Results" and Table 3) shows that this was achieved, and therefore, the differences observed are related to hormonal variations. That participating women carried copper intrauterine devices is a limitation to the study; they were accepted because there is evidence in the literature that intrauterine devices (IUDs) do not modify stradiol and progesterone plasma concentrations [16], in contrast to oral contraceptives, which do change these hormones concentrations in blood [17].

It is interesting that serum SHBG concentration consistently dropped until the fourth month of study, coinciding with a gradual increase of serum ceruloplasmin. Administration of 17-β-stradiol to Long-Evans-Hooded (LEC) rats induced increased serum copper and ceruloplasmin [18]. In postmenopausal women, treatment with 17-β-stradiol also induced an increase of serum ceruloplasmin [19]. These two studies coincide with the trend observed in the first 4 months of our study, but we do not have an explanation for the decrease observed in subsequent months.

It is also interesting that the post-hoc analysis applying the Bonferroni correction confirmed the differences between sexes and further revealed that GGT and activities were different in the two phases of the cycle ($P<0.05$). The influence of time of sampling should be further evaluated.

In summary, when healthy adult women and men undergo controlled chronic copper dosing, the variations of serum copper indicators and of serum SHBG and of liver function indicators are associated with the variations of estrogens/progesterone cycling. This should be considered when interpreting studies that use these indicators as outcomes of copper effects.

Acknowledgements Authors express their gratitude to Dr. Emilio Fernandez for his expert advice on the management of hormonal aspects that defined the groups and times of sampling. This study was funded by Fondecyt, project 1040979.

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