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Acute Copper and Ascorbic Acid Supplementation Inhibits Non-heme Iron Absorption in Humans

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Abstract The objective of the study is to determine the effect of copper (Cu) plus the reducing agent ascorbic acid (AA) on the absorption of non-heme iron (Fe). Experimental study with block design in which each subject was his own control. After signing an informed consent, 14 adult women using an effective method of contraception and negative pregnancy test received 0.5 mg Fe, as ferrous sulfate, alone or with Cu, as copper sulfate, plus ascorbic acid (AA/Cu 2/1 molar ratio) at 4/1; 6/1 and 8/1 Cu/Fe molar ratios as an aqueous solution on days 1, 2, 14, and 15 of the study. Fe absorption was assessed by erythrocyte incorporation of iron radioisotopes ^{55}Fe and ^{59}Fe. Geometric mean (range ± SD) absorption of Fe at 4/1 and 6/1 Cu/Fe molar ratios (and AA/Cu 2/1 molar ratio) and Fe alone was 57.4 % (35.7–92.1 %), 64.2 % (45.8–89.9 %), and 38.8 % (20.4–73.8 %), respectively (ANOVA for repeated measures $p < 0.001$; post hoc test Scheffé, $p < 0.05$). This is attributable to the enhancing effect of AA on non-heme Fe absorption; however, Fe absorption at Cu/Fe 8/1 molar ratio was 47.3 % (27.7–80.8) (p = NS compared with Fe alone). It was expected that Fe absorption would have been equal or greater than at 4/1 and 6/1 molar ratios. Copper in the presence of ascorbic acid inhibits non-heme Fe absorption at Cu/Fe 8/1 molar ratio.

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Introduction

Iron deficiency is the single most prevalent nutritional public health problem in the undeveloped world [1]. In this setting, it coexists with other micronutrient deficiencies, such as copper deficiency [2].

Combined supplementation with iron (Fe) and copper (Cu) is one of the strategies which might be used for preventing or treating these deficiencies. However, one of the most important factors that could influence the overall success of this strategy is the bioavailability of the mineral compound added to the supplement. A potential problem of combined supplementation with both Fe and Cu is a possible negative interaction between these minerals.

There is disagreeing information on whether Cu inhibits non-heme Fe uptake in Caco-2 cells [3–6]. We had observed that Cu, in the presence of ascorbic acid (AA), leads to a decrease in Fe uptake and that this inhibition is dose-dependent [4]. Two other studies have also described a reduction in Fe uptake with the addition of Cu [5, 6]. However, Zerounian and Linder found that Cu does not affect Fe uptake either in the presence or absence of AA [3].

In the only study that has been performed in apparently healthy humans, we demonstrated that Cu, as cupric sulfate, administered in an aqueous solution does not inhibit Fe bioavailability even at a Cu/Fe molar ratio of 8/1 [7]. Two questions arise when addressing this effect: Does Cu have to remain in a reduced state (Cu^{1+}) to be absorbed and thus inhibit Fe absorption? Does DMT1, the main non-heme iron transporter, have a significant role in Cu intestinal absorption?

The aim of the study was to determine the effect of reduced Cu (Cu1+) by AA on non-heme Fe absorption in human beings.

Subjects and Methods

Subjects

Fourteen apparently healthy women, between 34 and 47 years of age, were selected to participate in the study. None of the women were pregnant prior to the study, as confirmed by a negative test for human chorionic gonadotropin in urine, and all were using an intrauterine device as a method of contraception at the time of the study. All subjects were in apparent good health, and none had reported consuming vitamin or mineral supplements in the previous 6 months.

A written, informed consent was obtained from all the volunteers prior to the isotopic studies. The study protocol and consent form were approved by the Ethics Committee of the Institute of Nutrition and Food Technology of the University of Chile.

Isotopic Studies

Iron radioisotopes (^{59}Fe and ^{55}Fe) of high specific activity were used as tracers (Du Pont de Nemours, Wilmington, DE). Aqueous solutions containing Fe alone, as ferrous sulfate, were mixed with the radioisotopes immediately before administration to the subjects. A total amount of 100 mL of the labeled solutions containing 0.5 mg of Fe was administered to each subject. The radioisotope doses and administration protocols had been previously approved by the Chilean Commission of Nuclear Energy.

Cu, as cupric sulfate, and ascorbic acid (molar ratio 2/1 to copper) were given in gelatin capsules (number 0; Reutter).

Isotopically labeled solutions, Cu and AA capsules were consumed after an overnight fast, and no food or beverages other than water were allowed for the following 4 h.

The study design contemplated administration of four different treatments in an acute experimental crossover design, where Fe absorption was compared within the same subjects. The sequence of administration of the treatments was randomly assigned to the group. On day 1, all subjects received 0.5 mg of Fe alone labeled with 111 kBq ^{55}Fe (Cu/Fe molar ratio 0/1), and on day 2, they received 0.5 of Fe labeled with 37 kBq of ^{59}Fe plus 4.55 mg of Cu and 25.22 mg of AA (Cu/Fe molar ratio 8/1). A venous blood sample was obtained on day 14 to measure circulating radioactivity from isotope ingestion at days 1–2 and to determine the Fe status of the subjects. In addition, these blood samples also served as baseline values of the amount of ^{59}Fe and ^{55}Fe radioactivity in red blood cells for the next set of absorption studies (days 14–15). Subjects were then given 0.5 mg of Fe labeled with 111 kBq ^{55}Fe plus 2.28 mg of Cu and 12.64 mg of AA (Cu/Fe molar ratio 4/1). The following day (day 15), they received 0.5 of Fe labeled with 37 kBq of ^{59}Fe plus 3.41 mg of Cu and 18.90 mg of AA (Cu/Fe molar ratio 6/1). A final venous sample was obtained on day 28 to measure the increase in red blood cell radioactivity.

Blood Analyses

Hemoglobin (CELL-DYN 3200, ABBOTT Diagnostics, Abbott Park, IL), transferrin saturation [8], Zn-protoporphyrin (ZP Hematofluorometer model 206D, AVIV Biomedical Inc., Lakewood, NJ), and serum ferritin [9] were assessed to evaluate the Fe status of the subjects. Serum Cu was measured by atomic absorption spectrometry (model 2280; Perkin-Elmer and Analytic Sciences, Norwalk, CT).

Fe deficiency was defined as having a hemoglobin concentration >120 g/L together with at least two of the following: transferrin saturation <15 %, Zn-protoporphyrin >70 μg/dL RBC, and serum ferritin <12 μg/dL. Fe deficiency anemia was defined as having a hemoglobin concentration <120 g/L together with two abnormal indicators of Fe status. Cu deficiency was defined as serum Cu <80 μg/dL [10].

For the calculation of total radioactivity ingested, radioactivity was counted in sextuplicate from aliquots taken from each labeled solution. The measurement of venous blood radioactivity was performed from duplicate samples according to the technique of Eakins and Brown [11]. All samples were analyzed using a liquid scintillation counter (Tri-Carb 1500TR; Packard Instruments Co., Downers Grove, IL) allowing sufficient reading time that would lead to a counting error of <3 %. Radioactivity from the aliquots of labeled solutions and from the blood samples were counted simultaneously at the end of the study to avoid an error in the calculation of Fe absorption due to the decay which had occurred between administration of the isotopes and the absorption measurement 14 days later. In addition, the absorption of iron administered on days 14 and 15 was also corrected for the isotope which had been administered on days 1 and 2 by subtracting the radioactivity of the blood sample of day 14 from red blood cell radioactivity of day 28. The percentages of iron absorption were calculated on the basis of blood volumes estimated for height and weight [12] and assuming that 80 % of the radioisotope is incorporated into the erythrocyte [13]. This method is reproducible in our laboratory with a coefficient of variation of 5 %.

Statistical Methods

Because the percentages of Fe absorption and serum ferritin have skewed distributions, the values were first converted to their logarithms. The results were then retransformed to their

Table 1 General characteristics, iron and copper status of subjects

Age (years)	41.1 ± 4.4
Weight (kg)	65.4 ± 10.9
Height (m)	1.56 ± 0.1
Hemoglobin (g/L)	136 ± 6
Mean cell volume (fL)	91 ± 5
Zn-protoporphyrin (μg/dL RBC)	80.6 ± 25.7
Transferrin saturation (%)	27.7 ± 12.7
Serum ferritin (μg/L)[a]	19 (7–49)
Serum copper (μg/dL)	99.3 ± 29.7

Mean ± SD, except where noted

[a] Geometric mean ± range 1 SD

antilogarithms to recover the original units and then expressed as geometric means and ±1 SD ranges. An ANOVA for repeated measures (Statistica for Windows, release 4.5, StatSoft Inc., Tulsa, OK) was used to compare the absorption of iron from the four treatments administered within the study. All comparisons were done at the 5 % level of significance.

Sample Size Calculation

A sample size of nine subjects was calculated using the software PRIMER, version 3.02, option "power and simple size ANOVA". The sample size was calculated with an α level of 0.05, a power of 80 %, an expected residual standard deviation of three, four treatments and a minimum detectable difference of five percentage points of iron absorption. Fourteen volunteers were included for a possible loss of follow-up.

Results

The general characteristics and Fe and Cu status laboratory indicators of the volunteers are shown in Table 1.

The Fe and Cu nutritional status of most of the subjects who participated in these studies was normal. Only three women (21 %) presented Fe deficiency without anemia and two were Cu deficient.

The absorption of 0.5 mg of Fe alone or in combination with Cu and AA at different Cu/Fe molar ratios is shown in Table 2. Geometric mean (range ± 1 SD) absorption of Fe at 4/1 and 6/1 Cu/Fe molar ratios plus AA at 2/1 molar ratio to Cu, and Fe alone were 57.4 % (35.7–92.1 %), 64.2 % (45.8–89.9 %), and 38.8 % (20.4–73.8 %), respectively (ANOVA for repeated measures $p < 0.001$, post hoc test Scheffé, $p < 0.05$). This effect on iron absorption at 4/1 and 6/1 Cu/Fe molar ratios is attributable to the enhancing effect of AA on non-heme Fe absorption; however, Fe absorption at Cu/Fe 8/1 molar ratio with AA was 47.3 % (27.7 to 80.8) (p = NS compared with Fe alone).

The ratios of iron absorption were calculated by dividing the absorption of iron in the presence of Cu and AA by the absorption of Fe in the absence of Cu and AA, and they were shown as mean ± SD (Fig. 1).

Discussion

The main site of both Cu and Fe absorption is the duodenum [14]. Non-heme Fe uptake by the enterocyte is mediated mainly by divalent metal transporter-1 (DMT1), while the uptake of Cu occurs predominantly via the high affinity human Cu transporter 1 (Ctr1) [14, 15]. Both minerals must be reduced to be carried by these transporters [14, 16]. The process is mediated by the action of reductases localized in the apical membrane of the enterocyte [14, 16] and this effect is enhanced by the action of AA [17]. It is controversial whether and to what extent DMT1 is also able to transport Cu. Recent evidence shows that Cu is not a substrate for DMT1 [18] and Belgrade rats, a knockout for this transporter, do not develop Cu deficiency [19]. On the contrary, Arredondo et al. demonstrated that DMT1 carries Cu^{1+} and that Cu^{1+} is better substrate than Cu^{2+} for this transporter [20]. More recently, our group showed that antisense oligonucleotides used to decrease the expression of endogenous DMT1 or CTR1 diminished both Cu and Fe uptake in Caco-2 cells [21], demonstrating that both transporters are able to transport both minerals. However, it is well known that DMT1 has a higher affinity for Fe and CTR1 for Cu.

In the current study, we studied the absorption of 0.5 Fe, as ferrous sulfate, when Cu, as cupric sulfate, was added at either 4/1, 6/1 or 8/1 molar ratios to Fe plus AA at 2/1 molar ratio to Cu. The mean absorption of Fe given alone was comparable with previously published values [22]. There was a significant

Table 2 Effect of graded doses of copper (0–4.55 mg) and ascorbic acid (0–25.22 mg) on iron absorption of 0.5 mg of iron (n = 14 subjects)

	Iron absorption (%)			
Cu/Fe molar ratio	0:1	4:1*	6:1*	8:1*
Geometric mean (%)	38.8	57.4	64.2	47.3
Range ± 1 SD	(20.4–73.8)[a]	(35.7–92.1)[b,c]	(45.8–89.9)[b,c]	(27.7–80.8)[a]

One-way repeated measures ANOVA, $F = 11.9$, $p < 0.001$. Different letters indicate significant differences according to post hoc Scheffé test

*Plus ascorbic acid at 2/1 molar ratio to copper

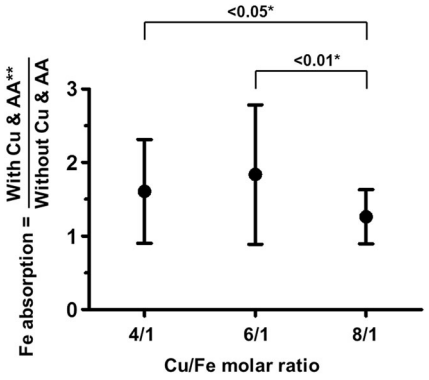

Fig. 1 The effect of various doses of copper and ascorbic acid on absorption of non-heme iron. The ratios were calculated by dividing the absorption of iron in the presence of copper and ascorbic acid by the absorption of iron alone. The *point* shows the geometric mean of ratios and *bar* shows the ±1 SD

increase in Fe absorption at 4/1 and 6/1 Cu/Fe molar ratios. This finding may be attributed to the promoting effect of AA on Fe absorption [23]. At 4/1 and 6/1 Cu/Fe molar ratios, the corresponding AA to Fe molar ratios were 8/1 and 12/1, respectively. However, Fe absorption at 8/1 Cu/Fe molar ratio was not significantly different compared with Fe absorption given alone (without Cu and AA); despite that AA to Fe molar ratio was 16/1. Since it has not been previously described whether high doses of AA inhibit Fe absorption, the most plausible explanation to this finding is that at this Cu/Fe ratio and in the presence of AA, there was a sufficient number of Cu^{1+} cations able to compete with Fe^{2+} for a common transporter or transporters. However, the inhibition of Fe absorption at this very high Cu/Fe molar ratio demonstrates that DMT1 is not a physiologically relevant Cu transporter. Previously, we have found that Cu, as cupric sulfate, given in solution without AA at Cu/Fe molar ratios up to 8/1 does not inhibit Fe absorption in humans [7]. This is most likely because the intestinal reductases were not sufficient to change a significant amount of Cu^{2+} to Cu^{1+}.

In conclusion, Cu administered in aqueous solution at 8/1 molar ratio to Fe in the presence of AA acid at 2/1 molar ratio to Cu voids the iron absorption enhancing effect of AA on non-heme Fe. This finding contributes to a better understanding the physiology of the absorption of both minerals and their interrelationships in nonphysiologic doses or in molar ratios non-typically found in the diet or in multi mineral supplements; however, these results do not have practical implications, given that supplements that contain both Fe and Cu, Fe is found in higher concentration than Cu because of their very different requirements [24].

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