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Administration of High Doses of Copper to Capuchin Monkeys Does Not Cause Liver Damage but Induces Transcriptional Activation of Hepatic Proliferative Responses[1–3]

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Abstract

Liver cells respond to copper loading upregulating protective mechanisms. However, to date, except for liver content, there are no good indicators that identify individuals with excess liver copper. We hypothesized that administering high doses of copper to young (5.5 mg Cu · kg^{-1} · d^{-1}) and adult (7.5 mg Cu · kg^{-1} · d^{-1}) capuchin monkeys would induce detectable liver damage. Study groups included adult monkeys (2 females, 2 males) 3–3.5 y old at enrollment treated with copper for 36 mo (ACu); age-matched controls (1 female, 3 males) that did not receive additional copper (AC); young monkeys (2 female, 2 males) treated from birth with copper for 36 mo (YCu); and young age-matched controls (2 female, 2 males) that did not receive additional copper (YC). We periodically assessed clinical, blood biochemical, and liver histological indicators and at 36 mo the hepatic mRNA abundance of *MT2a*, *APP*, *DMT1*, *CTR1*, *HGF*, *TGFβ*, and *NFκ* only in adult monkeys. After 36 mo, the liver copper concentration was 4–5 times greater in treated monkeys relative to controls. All monkeys remained healthy with normal routine serum biochemical indices and there was no evidence of liver tissue damage. Relative mRNA abundance of *HGF, TGFβ* and *NFκB* was significantly greater in ACu than in AC monkeys. In conclusion, capuchin monkeys exposed to copper at doses up to 50 times the current upper level enhanced expression of genes related to inflammation and injury without clinical, blood biochemical, or histological evidence of liver damage. J. Nutr. 142: 233–237, 2012.

Introduction

Potential risks associated with high chronic copper intake from foods and water have been a concern to health researchers and regulators. Gastrointestinal effects of acute exposure to copper in water were reported (1–5), leading to the revision of the guideline established by WHO for copper concentrations in water (6). However, the WHO expert committee at the time identified the need to clarify the safe range of chronic copper exposure in humans, which has proven to be a difficult task. Healthy adult women and men have been exposed to different copper doses over time (3,5,7), but because ethical constraints limit maximum doses and exposure periods, the levels tested have not exceeded the upper limit defined as safe for humans (8). Under these conditions, our studies have not been able to detect early, functionally important responses that might help identify individuals that are in the path of loading their liver with copper. We chose 3.8 μmol/g of copper in liver to define the potential risk from chronic copper exposure based on a previously published population risk assessment model that predicts potential frequencies of clinical and subclinical disease (9).

Data on the copper intake that might induce chronic adverse effects in humans are limited. Indian childhood cirrhosis (10–12) is a condition that related copper exposure to consumption of milk stored and/or heated in copper or copper alloy containers (13–15). There is an anecdotal case of a 26-y-old man who self-administered 30 mg/d of supplemental copper for 2 y and 60 mg/d for an additional year; he then presented liver failure and required liver transplantation (16,17). From 1900 to 1974, in the Austrian Tyrol, copper utensils were used to prepare infant foods; 138 infants and young children died from liver cirrhosis attributed to a high chronic copper exposure (18). No further cases were observed after communities abandoned the use of copper utensils (18). To what extent these cases represent copper toxicity in a normal population or whether these data were from a subgroup of genetically susceptible individuals is unknown.

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[3] Supplemental Table 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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Restrictions to humans in toxicity studies make animal models the only plausible way to address the assessment of copper effects at the whole body level. In a previous experiment, newborn rhesus monkeys (*Macaca mulatta*) received ~900 $\mu g \cdot kg^{-1} \cdot d^{-1}$ from birth to 6 mo of age and were assessed at 1, 4, and 6 mo of copper dosing. ^{67}Cu retention was 19.2 and 10.9% after 1 and 6 mo of copper treatment, respectively. These were compared with historical controls that retained 75% at age 2 mo. The liver copper concentration increased from 3.9 (controls) to 74.0 $\mu mol \cdot g\ dry\ tissue^{-1}$ at 1 mo of age and then decreased to 17.3 $\mu mol \cdot g\ dry\ tissue^{-1}$ at 6 mo, while still receiving the same dose of copper. No histological evidence of damage was detected during the study period (19).

The present study assessed the effects of chronic copper administration in healthy young and adult tufted capuchin monkeys. We hypothesized that copper dosing at 5.5 and 7.5 $mg \cdot kg^{-1} \cdot d^{-1}$ (in young and adult monkeys, respectively) would induce detectable liver damage after 12–24 mo of exposure. Clinical, blood biochemical, and histological indicators were periodically assessed. Because we did not detect changes after 24 mo, the study was prolonged for an additional 12 mo. At 36 mo, we still had not detected adverse clinical effects, including abnormal liver histology. We then assessed in the adult monkeys group the relative mRNA abundance of a set of genes related to copper metabolism and inflammatory and injury responses (20–24).

Materials and Methods

Design and copper dosing. All experiments were carried out humanely and with respect for alleviation of suffering following protocols approved by the institutional Animal Care Committee, Institute of Nutrition and Food Technology, University of Chile, which is based on the Declaration of Helsinki and follows the NIH Guide for the Care and Use of Laboratory Animals. Protocols were also approved by Pontificia Universidad Católica de Chile Institutional Animal Care Committee. Infant and adult tufted capuchin monkeys were obtained from the Catholic University Primate Center, Santiago, Chile, where the monkeys were housed.

Sixteen (8 healthy young and 8 healthy adult) monkeys were maintained indoors under the constant care of nursery and veterinary staff in temperature-controlled rooms with 12-h light cycles. Food and water were consumed ad libitum (25). Monkeys that showed normal clinical and blood biochemical values were randomly assigned to one of four groups (4 animals each): adult (3–3.5 y at enrollment) monkeys treated with copper gluconate for 36 mo (ACu[6]; 2 females, 2 males), age-matched controls (AC; 1 female, 3 male adult controls, matched by age, no copper treatment); young (newborn at enrollment) monkeys treated with copper for 36 mo (YCu; 2 female, 2 males), and young age-matched controls (YC; 2 female, 2 males) fed the same formula without copper.

Young monkeys were bottle fed since birth with a standard, commercially available fortified cow milk infant formula (Purita fortificada) providing 26% fat and 100 mg Fe, 5 mg Cu, and 50 mg Zn/L formula. Their diets consisted of fresh foods plus vitamin and mineral supplements following the norms of the Primate Center, as previously published (25). Copper dosing was set at 5.5 mg and 7.5 mg/d (as copper gluconate) in 1 or 2 doses to avoid acute manifestations (mainly salivation and loose stools). In adult monkeys, copper dosing started at 5 $mg\ Cu \cdot kg^{-1} \cdot d^{-1}$ and progressively increased to 7.5 $mg \cdot kg^{-1} \cdot d^{-1}$ over a 2-mo period; in young monkeys the initial dose was 3.5 and increased to 5.5 $mg\ Cu \cdot kg^{-1} \cdot d^{-1}$, also within 2 mo. Copper was administered for 3 y in all monkeys. In adult monkeys, copper was hidden in fruits, covered with caramel fudge or preserve, and visually supervised until consumed. Young monkeys received the copper in milk bottles, with the dose adjusted to the monkey's body weight every 2 wk. Fruits and vegetables were introduced to the diet at 4–6 mo of age; independent of this, young monkeys continued receiving a 200-mL bottle of milk that provided the daily copper dose during the whole study period.

Procedures and measures. Monthly health check-ups clinically evaluated monkeys (well being, activity, playing time, appetite), their weight, and length (crown-to-rump). Blood samples (3 and 5 mL of blood) were collected in young and adult monkeys, respectively, from the saphenous vein, after sedation with Ketamin (Ketaset, 10 $mg \cdot kg^{-1}$, Wyeth Ayerst) every second month during the first year and every third month thereafter. Liver was biopsied every third month during the first year and every 6 mo thereafter. Samples were obtained between 0830 and 0900 h after 4 h or overnight food deprivation in young and adult monkeys, respectively. In addition, a hair sample was obtained from the monkey's flank and kept at room temperature until analyzed. Hematological indicators, liver aminotransferases (serum aspartate aminotransferase, alanine aminotransferase, and GGT), and serum and hair copper concentrations were measured as previously described (25).

Liver samples were obtained surgically, initially from the left lobule and later from the right lobule, avoiding scar tissue. One biopsy was snap-frozen at −80°C and analyzed for total copper and iron concentrations as previously described (19). A second piece was fixed in 4% formaldehyde, dehydrated, and included in paraffin; 5-micron sections were stained with hematoxylin and eosin (to assess general hepatic architecture) and rhodamine (to visualize copper deposits distribution) following routine techniques; immune histochemical assessment used monoclonal antibodies, clone NCL-METALLO E9 for metallothionein (Novocastra Laboratories), and NCL-Ki67-MM1 for cell proliferation (any cell cycle state except G0, MM1, Novocastra Laboratories). A 3rd piece was fixed for transmission electron microscopy as previously described (26) and viewed with a Philips CM100 electron microscope operating at 80 kV.

Real-time RT-PCR analysis. After 24 mo of copper loading, young and adult experimental monkeys had liver copper concentrations 4–5 times greater than controls and biochemical and histological indicators remained negative. Immuno-histochemistry of liver biopsies revealed positive results with antibodies Ki67 and MT1, indicating a proliferative response and increased MT1 content, respectively. This led us to extend the protocol for an additional 12 mo, at the end of which we assessed in adult monkey liver biopsies the relative abundance of four transcripts encoding proteins related to copper uptake, storage, and metabolism: *MT2a* (26), *APP* (29), *DMT1* (30), and *CTR1* (31) and three proteins related to hepatic responses to injury: *HGF* (32,33), *TGFβ* (23), and *NFκ* (34). RNA from liver cells was extracted using the TRI Reagent kit (Ambion) according to the manufacturer's instructions. After that, RNA were treated with RNAse-Free DNAse Set (Qiagen) and reverse transcribed with Oligo-dT and Superscript II (Invitrogen). RT-PCR reactions were carried out as previously described (35). Primer sequences are shown in **Supplemental Table 1**. All primers were designed on the basis of human genes using the Primer Premier 5.0 software (Premier Biosoft International) and synthesized by Alpha DNA. The ortholog of human β-actin was used as reference to normalize the expression levels. Efficiency was determined for each sample and gene by LinRegPCR v7.5 as previously described (27). The quality of the PCR reactions was checked through analysis of the melting curves. Products were resolved by 2% agarose gel electrophoresis to confirm unique DNA fragments of expected size. Samples of each monkey were assayed in duplicate.

Statistical analysis. Analyses were conducted using SYSTAT 11. Comparisons were made between ACu and AC monkeys and YCu and YC monkeys but not between different age groups. Analysis was based on the group data collected at the different study times, assessed by age, treatment group, and time of exposure repeated-measures ANOVA. Because these analyses revealed no differences, results are not shown; ACu/AC and YCu/YC were then compared by two-sample *t* test at incorporation and at 6, 12, 24, and 36 mo. Because differences were not significant, only 36-mo data are presented in "Results." Differences were considered significant at $P < 0.05$. Histological and immunohistochemical results were analyzed by comparing serial sections of liver tissue obtained at different times of the study in a blind fashion. Ki67 and MT1

[6] Abbreviations used: AC, adult control (untreated) monkey; ACu, adult copper-treated monkey; GGT, gammaglutamyl transferase; YC, young control (untreated) monkey; YCu, young copper-treated monkey.

semiquantitative analysis was expressed as fold of the control. Variations in mRNA abundance were expressed as fold of control values normalized to β-actin. The ACu and AC groups were compared using the Mann-Whitney U Test, as described by Del Pozo et al. (28).

Results

Clinical aspects and diet. Monkeys remained healthy, maintained their customary activities and appetite, and there were no differences in food intake or body weight between experimental and control groups; in the young groups this included no differences in weight gain by age or time of exposure (data not shown). Growth (length and weights) was as expected and did not differ between YCu and YC or YCu and a comparison reference group (25) (data not shown). Milk bottles offered to young monkeys were ingested ~100%. In adult monkeys, daily records of leftovers (visually estimated) yielded ~90% consumption of copper doses during the 3-y period.

Blood biochemistry and copper in serum, liver, and hair. Results were first compared to values previously obtained in the reference group (25). At 36 mo, Fe nutrition indicators, liver enzymes, and serum metal concentrations were for all four groups within the range measured in the colony (**Table 1**). However, hemoglobin and mean corpuscular volume were significantly lower and free erythrocyte protoporphyrin was significantly greater in AC than in ACu mokeys; the YCu and YC groups did not differ in any of these variables. Liver aminotransferases did not differ between the adult groups, but GGT was significantly greater in YCu compared with YC monkeys. However, it remained lower than cases of hepatitis in the animal house in previous years. After 36 mo of copper supplementation, copper concentrations in liver and hair were significantly greater in the ACu and YCu groups than in the AC and YC groups, respectively (Table 1).

Histological studies. By light microscopy, the liver architecture and histology of adult and young experimental monkeys did not change over time, with no signs of hepatitis, changes in cell types, apoptosis, or appearance of fibrosis. Rhodamine staining was negative at all sampling times before 12 mo, when a few isolated periportal cells became positive. At 18 mo, patches of positive cells appeared around the portal tracts (**Fig. 1***A*), progressively increasing until 36 mo, when large patches of positive cells extended beyond the periportal areas (Fig. 1*B*) and also appeared in large numbers within the hepatic lobules (Fig. 1*C*). Rhodamine staining remained negative in control monkeys throughout the study. Transmission electron microscopy revealed electron-dense granules starting at 12 mo and progressively increasing in number over time, reaching a maximum at 30 mo. We interpreted this as possible copper lysosomes deposits (not shown). Mitochondria and the nuclei remained unchanged. There was evidence of nonspecific changes, with a moderate increase in smooth endoplasmic reticulum membranes over time, parallel to the appearance of some vesicles and collagen fibers in periportal areas.

Ki67 positive cells in biopsies from experimental and control monkeys in both age groups maintained a pattern similar to that observed for rhodamine positive cells at the different times of study (Fig. 1*D*); also, they showed a clear trend to increase over time in ACu and YCu monkeys. In ACu monkeys at 36 mo, the number of positive cells (counted in 10 random fields) was three times that of the AC group ($P < 0.05$). Beginning at 6 mo, MT1 monoclonal antibody also showed a progressive increase of positive cells. In the positive hepatocytes, the staining initially was positive only in the cytoplasm and later it also appeared in the nuclei (Fig. 1*E*,*F*).

Molecular markers at 36 mo. After 36 mo of treatment, the hepatic mRNA abundance of *NFκB*, *HGF*, and *TGFβ* was significantly greater in ACu than in AC monkeys (**Fig. 2**).

Discussion

The protocol successfully tested chronic copper intakes equivalent to 50 times the current upper limit, revealing no clinically detectable effects or significant biochemical or histological changes in young and adult tufted capuchin monkeys. Although liver histology remained unchanged, the increase in Ki67 positive

TABLE 1 Indices, blood chemistry, and serum, liver, and hair copper and iron concentrations in young and adult monkeys after 36 mo of control or copper treatment[1]

	AC	ACu	YC	YCu
Iron status				
Hemoglobin, *g/L*	156 ± 1.3*	147 ± 0.8	153 ± 3.4	154 ± 1.6
MCV, *%*	76.0 ± 0.2*	75.8 ± 0.2	72.3 ± 2.1	72.8 ± 1.1
WCC, $\times 10^6$/*L*	8.80 ± 0.3	6.80 ± 0.2	10.4 ± 0.5	9.40 ± 0.4
FEP, *μg/L RBC*	579 ± 16*	757 ± 26	507 ± 69	564 ± 39
Liver enzymes, *U/L*				
AST	22.4 ± 2.6	21.8 ± 2.4	18.4 ± 4.0	25.4 ± 3.5
ALT	24.5 ± 2.3	24.5 ± 2.0	18.7 ± 2.9	27.7 ± 2.5
GGT	20.2 ± 1.9	24.5 ± 1.8	27.2 ± 6.3*	43.1 ± 1.9
LDH	130 ± 16	118 ± 21	115 ± 24	101 ± 11
Tissue copper and iron concentrations				
Serum copper, *μmol/L*	846 ± 24.3*	714 ± 24.0	559 ± 31.5	768 ± 26.0
Hair copper, *nmol/g dry tissue*	116 ± 9.00*	234 ± 17.0	172 ± 14.0*	343 ± 42.0
Liver copper, *nmol/g dry tissue*	217 ± 52.0*	1160 ± 147	348 ± 79.0*	1270 ± 587
Hair Fe, *nmol/g dry tissue*	790 ± 300	710 ± 340	490 ± 76.0	520 ± 70.0
Liver Fe, *nmol/g dry tissue*	1780 ± 730	1230 ± 50.0	3210 ± 890	3040 ± 780

[1] Values are mean ± SEM, $n = 4$. *Different from corresponding copper-supplemented group, $P < 0.05$. AC, adult control (untreated) monkey; ACu, adult copper-treated monkey; ALT, alanine aminotransferase; AST, serum aspartate aminotransferase; FEP, free erythrocyte protoporphyrin; GGT, gammaglutamyl transferase; LDH, lactate dehydrogenase; MCV, mean corpuscular volume; WCC, total white cell count; YC, young control (untreated) monkey; YCu, young copper-treated monkey.

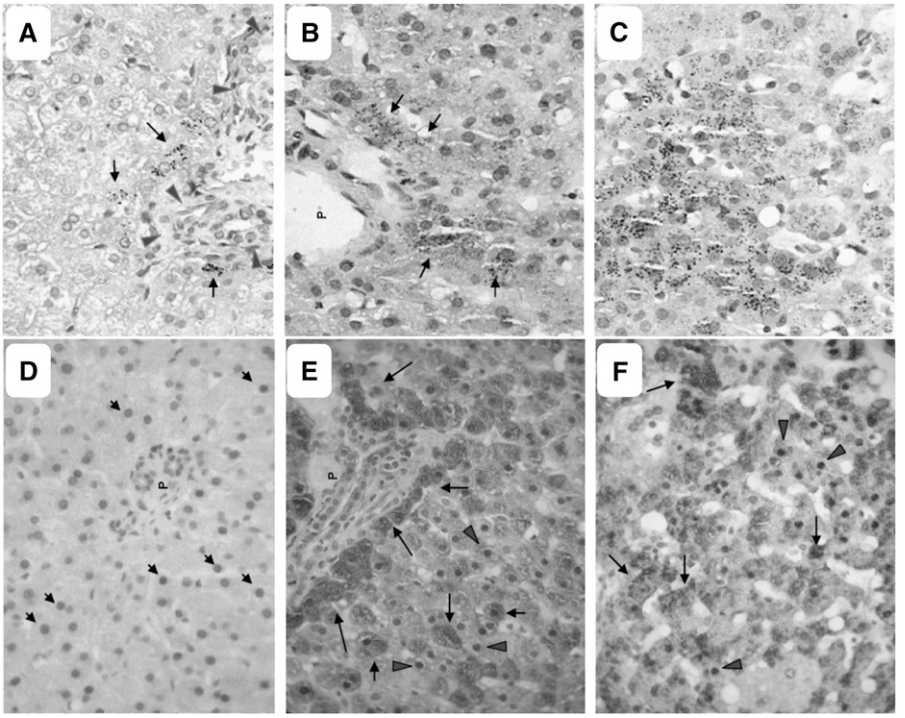

FIGURE 1 Liver biopsies of adult monkeys after 36 mo of copper treatment. Liver histology did not differ from the untreated adult group. Few periportal positive rhodamine cells were seen after 18 mo; arrows indicate positive rhodamine granules and arrowheads the limits of portal spaces (*A*). Abundant periportal positive staining was observed at 36 mo (arrows, p indicates the area of portal space; *B*). Abundant positive rhodamine granules were visible among hepatocytes at 36 mo (*C*). Ki67 positive nuclei (arrows) are close to a periportal area (*D*). MT1 positive cells were visible in a periportal area and within hepatocytes, respectively, at 36 mo; arrows indicate cells with positive cytoplasm and arrowheads positive nuclei (*E,F*). All photographs are 40×.

cells over time indicates that tissue proliferation was induced, an interesting finding considering that the basal liver status is nonproliferative (22). Proliferation is one of the inherent mechanisms for liver protection from oxidative damage (29–31).

Because the clinical, biochemical, and histological results were negative, measures of gene expression were important. Susceptibility to acute copper exposure has been related to factors such as species, age, and diet (5,7,32), but it is not known how MT changes after chronic excess copper exposure in humans. We selected genes related to proliferative responses to chemical agents and hepatectomy (23,31,33–35). Our results of increased Ki67 positive cells plus the upregulation of *HGF* and *NFκB* strongly suggest that these genes are also involved in the handling of excess copper in the absence of liver damage demonstrable by classical (biochemical and histological) techniques.

This study has the limitation of controlling copper intake, but not retention, because it was not possible to maintain chronic metabolic balance studies. Young monkeys received copper in milk bottles and leftovers were easily estimated; intakes were close to 100%. In the adult monkeys it was more difficult to measure copper intake; however, based on visual evaluations and daily recordings, most of the copper offered was ingested. We found greater liver and hair copper in both treated groups relative to their age-matched controls. It is worth noting that at 36 mo, the liver copper concentration was greater in the copper-treated groups than in controls by a factor of 4 in the adults and 5 in the young monkeys. We interpret our results as evidence that we succeeded in our objective to achieve liver copper loading without clinical or histological damage.

Liver damage was not demonstrated; however, indicators of iron status were compromised in the ACu group. Conversely, the YCu monkeys fed iron-fortified formula did not have compromised iron status. These results suggest that iron metabolism was indeed influenced by excess copper intake. We have previously reported in mammalian cell culture studies the interactions between copper and iron (36), but we did not find evidence that these relations occur in primates. Another limitation of the study was our inability to assay *CCS* mRNA expression due to the failure of the several primers assayed. Recent reports indicate that this copper *SOD* chaperone responds to copper deficiency in rats and mice (37).

We conclude that copper at the doses provided to capuchin monkeys did not induce toxic effects. Species-specific differences should be considered before extending these findings to humans.

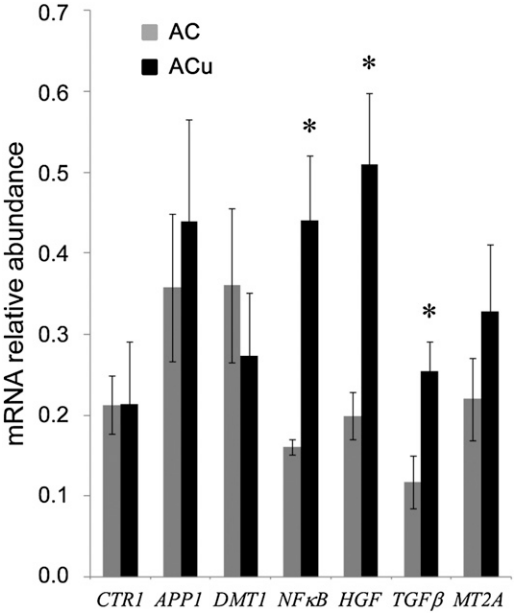

FIGURE 2 Hepatic mRNA levels of genes associated with copper metabolism and liver response to injury in adult monkeys after 36 mo of control or copper treatment. Genes related to copper metabolism: *APP1, DMT1, MT2A, CTR1*. Genes related to liver response to injury: *HGF, TGFβ*, and *NFκ*. The definitions of gene symbols are in Supplemental Table 1. Results were normalized to the abundance of the β-actin transcript and they are presented as a ratio between the value of each sample and the value of the highest measurement for each gene. *Different from AC, $P < 0.05$ (Mann-Whitney U statistic test).

Acknowledgments

M.A. and M.G. designed the protocol; M.A., M.O., F.C., L.P., H.N., and W.S. conducted the research; M.M., F.C., F.P., L.P., M.O., and M.A. analyzed data; M.A. and R.U. wrote the paper; and M.A. had primary responsibility for final content. All authors read and approved the final manuscript.

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