Effects of Moderate Copper Deficiency on Carbon Tetrachloride-Induced Hepatotoxicity in Rats (43220)

ROBERT A. DISILVESTRO*,[1] AND GARY P. CARLSON[†]

Department of Human Nutrition and Food Management, Ohio State University, Columbus, Ohio 43210 and Department of Pharmacology and Toxicology,[†] Purdue University, West Lafayette, Indiana 47907*

Abstract. **Extreme copper deficiency has been shown to enhance CCl_4-induced injury in rats. CCl_4 hepatotoxicity was studied in rats with copper deficiency moderated by limiting deficiency periods to 5 or 6 weeks, using minimally adequate dietary zinc and including a marginal copper diet. Also, housing some rats in groups of six, rather than individually, was found to moderate the effects of low copper intake. Weanling male rats were fed copper at either 6, 2, or 0.2 mg/kg diet (adequate, marginal, deficient). Copper-zinc superoxide dismutase activity levels for singly and group-housed marginal rats were 80% and 93%, respectively, of adequate values. Values for deficient rats were 35% (singles) and 47% (group). In singly housed rats, a CCl_4 dose of 400 μl/kg intraperitoneally increased serum sorbitol dehydrogenase activities, indicators of cell membrane hepatotoxicity, in inverse proportion to dietary copper. A lower dose (100 μl/kg) also produced smaller sorbitol dehydrogenase increases in adequate rats compared with deficients, but produced lowest increases in the marginals. The latter pattern also occurred in group-housed rats given the higher CCl_4 dose, but the difference for adequate and marginal rats was not significant. The higher CCl_4 dose, in singly housed rats, decreased liver glucose-6-phosphatase activities independently of copper intake. These activities are inversely proportional to microsomal lipid damage. In conclusion, moderate copper deficiency enhanced CCl_4 hepatotoxicity, but the effect depended on injury criteria, CCl_4 dose, and actual copper status as assessed by copper-zinc superoxide dismutase activities.** [P.S.E.B.M. 1991, Vol 197]

Copper deficiency is believed to render rats highly susceptible to oxygen radical injury (1–4). One reason is that copper deficiency produces low activity levels of Cu-Zn superoxide dismutase (SOD) (1), a cytosolic enzyme which eliminates the oxygen radical superoxide (5). In addition, microsomes from copper-deficient rats are highly susceptible to lipid peroxidation induced *in vitro* (2). Moreover, copper-deficient rats show low resistance to injury from oxidative stress such as hyperoxia and carbon tetrachloride (CCl_4) injection (3, 4). Currently, it is not clear to what degree copper function must be compromised before high susceptibility develops toward oxidant injury *in vivo*.

One approach to answering this question involves measuring oxidant toxicity in rats with varying degrees of copper deficiency. Lawrence and Jenkinson (4) found that severe copper deficiency enhanced CCl_4-induced elevation of expired pentane, a measure of lipid peroxidation. Growing rats were fed low copper for 8 to 10 weeks and displayed liver SOD activity levels about 20% of control values (4). The difference in Cu-Zn SOD activities might have been even greater since samples may have included some mitochondrial Mn SOD.

The present study determined the effects of more moderate copper deficiency states on CCl_4 hepatotoxicity. Copper deficiency was made more moderate with shorter diet feeding periods, by using lower dietary levels of zinc, an element which antagonizes copper absorption (6), and by including rats fed a diet marginal, but not deficient, in copper. In addition, the study presented here found that housing some rats in groups, rather than individually, also moderated copper deficiency.

This study evaluated two indices of CCl_4 injury,

[1] To whom requests for reprints should be addressed.

Received October 5, 1990. [P.S.E.B.M. 1991, Vol 197]
Accepted December 21, 1990.

0037-9727/91/1971-0032$3.00/0

each considered an index for a different stage of the injury process. CCl_4 hepatotoxicity is thought to occur in two stages (7). In one stage, microsomal cytochrome P-450 activates CCl_4 to free radicals which directly damage those organelles. In another stage, damage spreads, eventually leading to hepatocyte death and loss of cell contents. In the present study, liver microsomal glucose-6-phosphatase was used to assess microsomal membrane injury because it provides a general indicator for damage to these membranes (7). Liver cell leakage was evaluated as serum activity levels of the hepatic enzyme sorbitol dehydrogenase (SDH), the most sensitive of measurements commonly used for this purpose (8).

Materials and Methods

Animals and Diets. Weanling male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN), housed in stainless steel cages, and given feed and deionized water *ad libitum*. The diet, prepared by ICN Biochemicals (Cleveland, OH), was similar to that used by Mills and Murray (9). Different amounts of zinc and iron were added (10 mg/kg zinc as zinc chloride and 35 mg/kg iron as ferrous sulfate). Total zinc and copper contents of the diet, reported in Results, were analyzed by wet ashing and atomic absorption spectrometry. For marginal and adequate diets, copper was added at 2 or 6 mg/kg, respectively, as copper sulfate.

Rats were fed the diets for 5 or 6 weeks as noted in the tables. CCl_4 injections (100 or 400 μl/kg, diluted in corn oil 1:1, v:v) were given intraperitoneally 24 hr before the rats were decapitated.

Copper Enzyme Assays. Ceruloplasmin activity was measured in serum stored for up to 4 days at 4°C by oxidation of *p*-phenylenediamine as described by Rice (10). A unit was designated as the change in absorbance at 540 nm/15 min. Cu-Zn SOD activity was assessed in livers stored frozen at −20°C for up to 2 weeks. Livers were homogenized in five volumes of phosphate-buffered saline using a Polytron Homogenizer from Brinkman Instruments, Inc. (Westbury, NY). Mitochondrial Mn SOD activity was eliminated in 1-ml portions of the homogenates by adding 0.4 ml of ethanol:chloroform (11) followed by microcentrifugation at 15,600 *g*. Supernatant Cu-Zn SOD activity was quantitated by the modified pyrogallol assay of Prohaska (11). The blank rate change in absorbance at 320 nm was 0.016/30 sec. A unit was the amount of sample giving 50% inhibition of the blank rate.

Hepatotoxicity Measurements. Hepatic microsomal glucose-6-phosphatase activities were determined in freshly removed livers by a standard method (12). Serum SDH activity levels of the hepatic enzyme SDH were assessed spectrophotometrically using fructose as the substrate (8).

Statistical Analysis. The effects of dietary copper variations on parameters of copper status were evaluated by a one-way analysis of variance (ANOVA) followed by a least significant differences analysis (LSD). Interacting effects of dietary copper with CCl_4 injections on hepatotoxicity measurements were evaluated by two-way ANOVA followed by LSD.

Results

Dietary zinc content, a potential influence on copper status, was 15 mg/kg. The previous study on copper deficiency and CCl_4 by Lawrence and Jenkinson (4) used 50 mg/kg zinc as $ZnCO_3$ plus whatever zinc was present in the protein source.

Three different dietary copper levels produced three different degrees of copper status in individually housed rats (Table I). Cu-Zn SOD activities in the marginal rats were 80% of adequate values, while the value for deficients was 36% of that for the adequates. Consistent with the goals of this study, liver Cu-Zn SOD activities for the marginal and deficient rats were both a higher percentage of adequate values than produced in deficient rats by Lawrence and Jenkinson (4). The same could be said for ceruloplasmin, a common means of assessing copper status (6). However, a comparison of ceruloplasmin activities for the deficient rats may be of little use since the readings were so low in both studies.

CCl_4 injection at 100 or 400 μl/kg did not appear to affect liver Cu-Zn SOD activity levels (data not shown). CCl_4-treated rats did tend to show lower activity per gram of liver than did nontreated rats, but this resulted from liver hypertrophy. CCl_4 did not lower Cu-Zn SOD activities when expressed per liver or per ethanol:chloroform extracted protein.

The higher CCl_4 dose increased serum SDH activities in inverse proportion to copper intake and liver Cu-Zn SOD activities (Table II). Serum SDH activities reflect leakage of hepatic enzymes into the serum (8). The lower dose also produced higher SDH increases in

Table I. Effects of Dietary Copper on Body Weight, Hepatic Cu-Zn SOD, and Serum Ceruloplasmin Activities[a]

Dietary copper	Body weight (g)	Ceruloplasmin (units/dl)	Cu-Zn SOD (units/g)
Adequate (6 ppm)	256 ± 6a	72 ± 3a	17,111 ± 444a
Marginal (2 ppm)	257 ± 9a	20 ± 3;	13,791 ± 1,042b
Deficient (0.2 ppm)	222 ± 13b	3 ± 1c	6,141 ± 260c

[a] Values are mean ± SE from five weanling rats fed diets for 5 weeks. Values in the same column with different letters were significantly different ($P < 0.01$, ANOVA followed by LSD).

Table II. Effects of Dietary Copper and CCl_4 on Serum SDH Activity Levels[a]

Dietary copper	SDH (units/dl)		
	CCl_4 dose (μl/kg)		
	0	100	400
Adequate	20 ± 10a	680 ± 50c	1980 ± 170f
Marginal	10 ± 10a	460 ± 80d	3100 ± 170g
Deficient	130 ± 70b	2650 ± 250e	4540 ± 430h

[a] Values are mean ± SE for five rats. Data were derived from the rats used for Table 1 plus CCl_4-injected rats from the same dietary treatment groups. CCl_4 was injected intraperitoneally 24 hr before sacrifice. Values with different letters were significantly different ($P < 0.01$, ANOVA followed by LSD).

Table III. Effects of Dietary Copper and CCl_4 on Hepatic Microsomal Glucose-6-Phosphatase Activities[a]

Dietary copper	Glucose-6-phosphatase activities (μmol PO_4/g liver/min)	
	CCl_4 Dose	
	0 μl/kg	400 μl/kg
Adequate	31.9 ± 2.8a	20.4 ± 1.1b
Marginal	34.4 ± 3.0a	24.8 ± 1.8b
Deficient	35.5 ± 4.2a	21.2 ± 1.0b

[a] Values are the mean ± SE from five rats also used in Table II. Values with different letters were significantly different ($P < 0.01$, ANOVA followed by LSD).

Table IV. Body Weight, Serum Ceruloplasmin, and Hepatic Cu-Zn SOD Activity Levels in Rats Housed in Groups of Six[a]

Treatment	Body weight (g)	Ceruloplasmin (units/dl)	Cu-Zn SOD (units/g)
Copper-adequate	288 ± 11a	64 ± 7a	18,175 ± 622a
Copper-marginal	284 ± 8a	18 ± 4b	16,875 ± 581b
Copper-deficient	263 ± 22a	7 ± 3c	8,540 ± 407c

[a] Values are mean ± SE from rats housed in groups of six and fed the diets for 6 weeks. Values with different letters were significantly different from values in the same column ($P < 0.01$, ANOVA followed by LSD).

Table V. Effects of Dietary Copper and CCl_4 on SDH Activity Levels in Rats Housed in Groups of Six[a]

Dietary copper	SDH (units/dl serum)	
	CCl_4 Dose	
	0 μl/kg	400 μl/kg
Copper-adequate	147 ± 20a	1716 ± 312b
Copper-marginal	100 ± 11a	1413 ± 413b
Copper-deficient	125 ± 14a	3897 ± 783c

[a] Values are mean ± SE for five rats. Data were derived from the same rats used for Table IV plus CCl_4-treated rats from the same dietary groups. CCl_4 was injected intraperitoneally 24 hr before sacrifice. Values with different letters are significantly different from one another ($P < 0.01$, ANOVA followed by LSD).

deficient rats compared with adequates. However, the smallest increases occurred in the marginal rats (Table II).

Microsomal glucose-6-phosphatase activities were decreased by the higher CCl_4 dose (Table III). Copper status did not influence this effect of CCl_4. Glucose-6-phosphatase activities are inversely proportional to microsomal lipid peroxidation and destruction of endoplasmic reticulum membranes (7).

In a subsequent experiment, weanling rats were fed one of three copper levels for 6 weeks, but were housed in groups of six, rather than individually. Rats fed marginal and deficient diets showed mean Cu-Zn SOD activities that were 93% and 47% of the adequate value, respectively (Table IV). These percentages were significantly higher than those obtained with the deficient and marginal diets in the individually housed rats ($P < 0.01$, ANOVA followed by LSD). In contrast to results for singly housed rats, body weights for group-housed-deficient rats were not significantly different from those of the adequates. However, dietary copper had a profound effect on ceruloplasmin activities under both housing conditions.

The higher CCl_4 dose affected serum SDH in the group-housed rats in a fashion resembling that observed for the lower dose in singly housed rats (Table II and V). In both cases, CCl_4 had the greatest effect on rats fed the deficient diet, and the least on rats fed the marginal diet. However, the group-housed marginal and adequate rats showed no statistical difference.

Discussion

Copper intake had no impact on one index of CCl_4-induced hepatic microsomal injury (Table III), but profoundly affected serum SDH levels, an index of liver cell leakage (Tables II and V). The first result is consistent with the lack of inhibition by Cu-Zn SOD *in vitro* of lipid peroxidation resulting from the interaction of CCl_4 and cytochrome P-450 (13). In addition, microsomes from copper-deficient rats show normal formation of carbon-centered radicals in response to NADPH *in vitro* (14). On the other hand, microsomes from copper-deficient rats show high sensitivity to NADPH-induced oxygen radical mediated lipid peroxidation (2). This increased sensitivity *in vitro* must not correspond to increased sensitivity to damage *in vivo* for the particular injury model and copper status used here.

A large difference in CCl_4 toxicity, based on SDH readings, occurred with just a 2-fold difference in Cu-Zn SOD activities for group-housed, copper-deficient rats compared with adequates (Tables IV and V). CCl_4 also produced more injury in singly housed, copper-marginal rats than in adequates (Table II). In this case,

Cu-Zn SOD activities in the former were only 20% lower than values for the latter (Table I). In fact, if all data from group and singly housed rats were combined, SDH levels after injection of 400 μl/kg CCl_4 correlated inversely with Cu-Zn SOD values (Pearson correlation coefficient = 0.94). This correlation indicated that differences in oxidant-induced hepatotoxicity can occur over a range of differences in copper status as reflected by liver Cu-Zn SOD activities.

The effects of copper status on CCl_4 toxicity may or may not actually result from variations in liver Cu-Zn SOD activities. In bacteria, paraquat, a superoxide generator, enhances CCl_4 toxicity (15). However, liver superoxide production in rats not treated with an exogenous superoxide generator may not be sufficient to influence CCl_4 poisoning, even in copper-deficient rats. Possibly, some effect of copper status on lipid metabolism (16) could be responsible for the effects on CCl_4 toxicity.

The mechanism which causes marginal copper status in some instances to offer protection against CCl_4 hepatotoxicity requires elucidation. The protective mechanism was either not present or overridden by other factors in group and singly housed copper-deficient rats that showed high susceptibility to CCl_4 liver injury (Tables II and V). Similarly, the mechanism was not sufficient to prevent singly housed, marginal rats from showing enhanced injury formation with the higher CCl_4 dose (Table II).

Housing rats in groups, rather than individually, definitely influenced the impact of low copper diets on Cu-Zn SOD activities and susceptibility to hepatotoxicity. Perhaps, the group-housed rats ingested copper from the other animals' fur or feces, particularly during the early feeding stages.

The results of this study concerning copper status and CCl_4-induced injury apply only to the effects of sublethal, acute CCl_4 treatments. The doses, given here as single injections, produced no mortality in rats from any dietary group. The higher dose was about 15% of that which kills nearly all extremely copper-deficient rats while sparing most adequates (4).

Dietary copper restriction enhanced CCl_4 hepatotoxicity even when liver Cu-Zn SOD activities were only 20% lower than values from copper-adequate rats. However, in some circumstances, marginal copper status actually offered some protection against this toxicity.

This work was supported by National Institutes of Health First Award DK39604-01.

The authors gratefully acknowledge the fine technical assistance of Kathryn Madden, Elizabeth A. David, Joy Fredericci and Tammy Foltz.

1. Chung KC, Romero M, Tinker D, Keen CL, Amemiya K, Rucker R. Role of copper in the regulation and accumulation of superoxide dismutase and metallothionein in rat liver. J Nutr **118**:859–864, 1988.
2. Davies NT, Sarkozy P. The effects of copper deficiency on lipid peroxidation in rat liver microsomes. In: Mills CF, Bremner I, Chesters JK, Eds. Trace Elements in Man and Animals. TEMA 5. Farnham Royal, UK: Commonwealth Agricultural Bureaux, pp39–42, 1985.
3. Jenkinson SG, Lawrence RA, Grafton WD, Gregory PE, McKinney MA. Enhanced pulmonary toxicity in copper-deficient rats exposed to hyperoxia. Fundam Appl Toxicol **4**:170–177, 1984.
4. Lawrence RA, Jenkinson SG. Effects of copper deficiency on carbon tetrachloride-induced lipid peroxidation. J Clin Lab Med **109**:134–140, 1987.
5. Hassan HM. Biosynthesis and regulation of superoxide dismutases. Free Radic Biol Med **5**:377–385, 1988.
6. Cousins RJ. Absorption, transport, and hepatic metabolism of copper and zinc: Special reference to metallothionein and ceruloplasmin. Physiol Rev **65**:238–309, 1985.
7. Recknagel RO, Glende EA. The carbon tetrachloride hepatotoxicity model: Free radicals and calcium homeostasis. In: Miquel J, Quintanilha AT, Weber H, Eds. Handbook of Free Radicals and Antioxidants in Biomedicine. Boca Raton, FL: CRC Press, Vol **3**: pp3–16, 1989.
8. Korsrud GO, Grice HC, McLaughlan JM. Sensitivity of several serum enzymes in detecting carbon tetrachloride-induced liver damage in rats. Toxicol Appl Pharmacol **22**:474–483, 1972.
9. Mills CF, Murray GR. The preparation of a semisynthetic diet low in copper for copper deficiency studies with rats. J Sci Food Agr **11**:547–552, 1961.
10. Rice EW. Standardization of ceruloplasmin activity in terms of international enzyme units. Anal Biochem **3**:452–456, 1962.
11. Prohaska J. Changes in tissue growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase subsequent to dietary or genetic copper deficiency in mice. J Nutr **113**:2048–2058, 1983.
12. Harper AE. Glucose-6-phosphatase. In: Bergmeyer HU, Ed. Methods of Enzymatic Analysis. New York: Academic Press, pp788–792, 1965.
13. Ekstrom G, Ingelman-Sundberg M. Mechanisms of lipid peroxidation dependent on cytochrome P-450 LM2. Eur J Biochem **158**:195–201, 1986.
14. Kubow S, Bray TM, Bettger WJ. Effects of dietary zinc and copper on free radical production in rat lung and liver. Can J Physiol Pharmacol **64**:1281–1285, 1986.
15. Yamamoto H, Nagano T, Hirobe M. Carbon tetrachloride toxicity on Escherichia coli exacerbated by superoxide. J Biol Chem **263**:12224–12227, 1988.
16. Gallagher CH, Reeve VE. Copper deficiency in the rat. Effect on liver and brain lipids. Aust J Exp Biol Med Sci **49**:453–461, 1971.