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Development of Copper Deficiency in Rats Fed Fructose or Starch: Weekly Measurements of Copper Indices in Blood (42232)

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Abstract. Copper deficiency was induced in weanling rats fed diets whose sole source of carbohydrates was starch or fructose for 7 weeks. Conventional parameters of copper status, plasma copper concentrations, ceruloplasmin activity, and erythrocyte superoxide dismutase (SOD) activity were longitudinally monitored weekly to follow the development of the deficiency and to correlate these indices with the degree of severity of the deficiency. Although 30% of the rats fed a copper-deficient fructose diet died and no deaths occurred in rats fed the copper-deficient starch diet, plasma copper, ceruloplasmin, and SOD activities were reduced to a similar extent in all rats fed copper-deficient diets regardless of the type of dietary carbohydrate. Thus, none of the indices used accurately reflected the greater degree of deficiency or mortality in rats fed the fructose diet deficient in copper. The results of the present study underscore the need for more sensitive tests or alternative parameters to assess copper status in living animals. © 1986 Society for Experimental Biology and Medicine.

We have recently demonstrated that signs of copper deficiency in rats are aggravated by feeding diets containing fructose as the sole source of carbohydrate as compared to diets containing starch (1–6). In those studies, the more severe deficiency in rats fed fructose as compared to rats fed starch was verified by measuring hepatic copper concentration (1, 2) and hepatic and renal superoxide dismutase (SOD) activity (5). In all previous studies, tissue measurements of copper indices were carried out at time of sacrifice, usually after at least 6 weeks of feeding the deficient diet (1–6). To determine the extent of copper deficiency in living animals noninvasive techniques must be used. Plasma ceruloplasmin activity is nondetectable after feeding any copper-deficient diet (1–5) and, therefore, is not a suitable parameter to differentiate degrees of severity of copper deficiency. Although serum copper levels were found to be significantly lower in copper-deficient rats fed fructose than in those fed starch, the absolute levels were too low to be of physiological importance (2). After the fourth week of being fed a copper-deficient diet, a high percentage of deaths occurred in the group of rats fed fructose (1, 2).

In practical terms, there is need for monitoring the development of copper deficiency in fructose fed rats without sacrificing them. This would permit prediction of deaths and characterization of different stages of copper deficiency. In addition, defining various degrees of deficiency would allow comparisons of experiments, both intra and interlaboratories. The information might also be relevant to assessing copper status in humans.

Materials and Methods. To minimize variations in copper indices at the start of the experiment, male Sprague–Dawley weanling rats (MA Laboratory animals, Walkersville, Md.) from the same litter were randomly divided into four separate groups according to type of dietary carbohydrates and copper levels.

Group 1	cornstarch, copper deficient (26 rats)
Group 2	cornstarch, copper supplemented (26 rats)
Group 3	fructose, copper deficient (40 rats)
Group 4	fructose, copper supplemented (26 rats)

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TABLE I. BODY WEIGHT (g)

Weeks on diet	Fructose −Cu	Starch −Cu	Fructose +Cu	Starch +Cu
0	51 ± 1	51 ± 2	51 ± 1	50 ± 2
1	79 ± 2	85 ± 3	83 ± 2	88 ± 2
2	121 ± 2	131 ± 2	129 ± 2	135 ± 2
3	158 ± 2	178 ± 2	172 ± 2	176 ± 3
4	177 ± 4	212 ± 3	204 ± 3	210 ± 4
5	202 ± 5	247 ± 3	243 ± 4	252 ± 5
6	208 ± 6	296 ± 5	266 ± 4	274 ± 4
7	208 ± 8	284 ± 7	276 ± 7	285 ± 7

Note. Means ± SEM of five rats per group.

ANOVA

Copper × Carbohydrate $P < 0.0001$

All diets contained (g/kg diet) 622 carbohydrate, 200 egg white, 95 corn oil, 30 nonnutritive fiber (cellulose), 35 AIN-76 salt mix (copper omitted), 10 vitamin mix AIN 76A supplemented with 2 mg biotin and 2.7 choline bitartrate. In contrast to previous studies copper carbonate was added to the copper-deficient mineral mixture instead of the drinking water. The copper-supplemented diets contained 6 μg Cu/g diet, and the copper-deficient diets contained 0.6 μg Cu/g diet as native copper as determined by atomic absorption spectrophotometry. To assure accuracy, Certified Standard Reference Materials were analyzed along with the diets.

Animals were weighed weekly. Starting on the first week and ending on the seventh week of the study, indices of copper status were measured weekly in blood obtained from five rats in each group. Except for the group of 40 rats fed the fructose diet deficient in copper, this protocol necessitated the use of the same five rats during the first and sixth; and second and seventh weeks. At all other times none of the rats were bled more than once during the study. Blood (0.5–1.0 ml) was collected from the tip of the tail in heparinized tubes and centrifuged. Copper concentration by graphite furnace atomic absorption spectrophotometry (7) and ceruloplasmin activity in plasma (8) and SOD activity in red blood cells (9) were determined. Plasma copper, ceruloplasmin activity, and erythrocyte SOD were also measured at time 0 from six weanling rats sacrificed at the beginning of the experiment. At the end of the seventh week, rats were anesthetized by sodium pentobarbital injection and blood was withdrawn from their abdominal aorta. Livers were removed, dried in a vacuum oven, and digested by a method combining dry heat and acid, and the concentration of hepatic copper was measured by atomic absorption spectrophotometry.

Results. Rats began dying after the fructose diet deficient in copper was fed for 4 weeks. By the seventh week, 12 rats fed this diet had died, while none of the copper-deficient, starch-fed rats died.

Growth rate was not affected by the type of dietary carbohydrates in copper supplemented rats (Table I). Differences in growth rates between copper-deficient and supplemented controls fed starch were not significant. The interaction between carbohydrate and copper level was significant for weeks 3 to 7.

Plasma copper concentration was significantly reduced by copper deficiency from the first week of the experiment and remained low throughout the study (Table II). Plasma copper levels were not affected by the type of dietary carbohydrate in the copper-deficient rats. In contrast, in copper-supplemented rats, although not significant at all weekly intervals, plasma copper was reduced by fructose when compared to starch.

Ceruloplasmin activity was undetectable in plasma of rats fed the copper-deficient diets for 7 days regardless of the type of dietary carbohydrate (Table III). In copper-supplemented controls, ceruloplasmin activity was significantly reduced by feeding of fructose as com-

TABLE II. PLASMA COPPER (μg/100 ml)

Weeks on diet	Fructose −Cu	Starch −Cu	Fructose +Cu	Starch +Cu
0		84 ± 2		
1	6 ± 2	8 ± 3	66 ± 2	69 ± 2
2	4 ± 1	4 ± 1	49 ± 5	57 ± 4
3	3 ± 1	3 ± 1	42 ± 8	68 ± 2
4	4 ± 2	3 ± 1	50 ± 11	67 ± 3
5	3 ± 1	7 ± 2	59 ± 15	89 ± 5
6	3 ± 0.2	3 ± 0.2	61 ± 11	73 ± 4
7	4 ± 0.7	5 ± 0.9	43 ± 19[a]	104 ± 5[b]

Note. Means ± SEM of five rats per group except for time 0 where $n = 6$.

[a,b] Numbers within a row not sharing a common superscript are significantly different ($P < 0.05$) by Student's t test.

TABLE III. PLASMA CERULOPLASMIN ACTIVITY (U/l)

Weeks on diet	Fructose −Cu	Starch −Cu	Fructose +Cu	Starch +Cu
0		73 ± 6		
1	ND[a]	ND	40 ± 2	45 ± 0.03
2	ND	ND	44 ± 19	84 ± 7
3	ND	ND	48 ± 15[b]	92 ± 4[c]
4	ND	ND	61 ± 9[b]	106 ± 1[c]
5	ND	ND	61 ± 25	113 ± 6
6	ND	ND	76 ± 19	100 ± 9
7	ND	ND	46 ± 16[b]	133 ± 8[c]

Note. Means ± SEM of five rats per group except for time 0 where $n = 6$.

[a] Nondetectable.

[b,c] Numbers within a row not sharing a common superscript are significantly different ($P < 0.05$) by Student's t test.

pared to starch at three of the seven sampling periods. Ceruloplasmin was undetectable in some copper-supplemented rats fed fructose.

Statistical analysis was not required to show that all copper-deficient rats had lower levels of plasma copper and ceruloplasmin activity than their copper-supplemented controls. Therefore, within a copper level the copper-supplemented controls fed the two carbohydrates were compared by Student's t test.

Erythrocyte SOD activity was reduced by copper deficiency when compared to copper-supplemented controls (Table IV). SOD activity decreased as the experiment progressed and differences due to age (weeks) were highly significant. Erythrocyte SOD was not altered by type of dietary carbohydrate in copper-deficient or in copper-adequate rats. A significant interaction between carbohydrate source and age (week) was observed. In weeks 3, 5, and 7 fructose appeared to decrease SOD activity in the supplemented rats.

Concentration of hepatic copper was significantly decreased by fructose feeding in both copper-deficient and supplemented rats when compared to starch feeding (Table V).

Discussion. Regardless of the type of dietary carbohydrate, all rats fed the low copper diets developed a severe copper deficiency that was evidenced from the first week of the study by low levels of plasma copper, ceruloplasmin activity, and erythrocyte SOD activity. Thus, these measurements can be used to verify a general state of copper deficiency. However,

TABLE IV. ERYTHROCYTE SOD (U/ml PACKED RED CELLS)

Weeks on diet	Fructose −Cu	Starch −Cu	Fructose +Cu	Starch +Cu
0		438 ± 54		
1	186 ± 21	132 ± 29	204 ± 33	238 ± 39
2	158 ± 39	180 ± 24	311 ± 36	247 ± 49
3	133 ± 18	158 ± 5	158 ± 5	440 ± 26
4	32 ± 9	38 ± 9	292 ± 12	294 ± 14
5	ND	25 ± 6	293 ± 30	396 ± 22
6	58 ± 10	62 ± 9	372 ± 42	394 ± 19
7	23 ± 2	19 ± 8	157 ± 45	317 ± 19

Note. Means ± SEM of five rats per group except for time 0 where $n = 6$.

ANOVA[a]	$P <$
Cu	0.001
CHO	NS
Cu × CHO	NS
Week	0.001
Cu × Week	0.001
CHO × Week	0.04
CHO × Cu × Week	NS

[a] 2 × 2 × 7 Analysis of variance.

as serum copper concentration, SOD and ceruloplasmin activities were similar in all copper-deficient rats, these three parameters alone were not sufficient to demonstrate that feeding fructose aggravated the deficiency as compared to feeding starch. The more severe copper deficiency in rats fed the fructose diet as compared to rats fed starch was demonstrated by (i) reduced body weight and by (ii) paler appearance of ears, nose, paws, and eyes, which was readily distinguishable from rats fed

TABLE V. HEPATIC COPPER CONCENTRATION

	Fructose −Cu	Starch −Cu	Fructose +Cu	Starch +Cu
			μg Cu/g	
Wet weight	0.8 ± 0.06	1.1 ± 0.07	3.4 ± 0.6	4.3 ± 0.1
Dry weight	2.9 ± 0.2	3.6 ± 0.2	11.9 ± 0.9	13.8 ± 0.4

Note. Means ± SEM of five rats per group.

ANOVA[a]	μg Cu/g wet wt $P <$	μg Cu/g dry wt $P <$
Cu	0.001	0.016
CHO	0.008	0.0001
Cu × CHO	0.05	0.18

[a] A 2 × 2 Analysis of variance.

starch. In addition, the high incidence of mortality (30%) occurring only in copper-deficient rats fed fructose and reduced hepatic copper concentrations demonstrated that the fructose diet did result in a more severe deficiency when compared to starch.

In a recent human study, ingestion of a low copper diet supplying 20% of the calories from fructose as compared to starch did not alter ceruloplasmin activity or serum copper concentrations but did significantly reduce SOD concentration and activity of erythrocytes (10). During the study, 4 of the 24 subjects exhibited heart-related abnormalities (10). In another human study, Klevay *et al.* (11) have reported that in a healthy young man fed a low copper diet (0.83 mg/day), plasma copper and ceruloplasmin were not as sensitive as erythrocyte SOD and plasma cholesterol in establishing reduced copper status. Thus, in humans (10) but not in rats, erythrocyte SOD activity provided an indicator of difference in copper deficiency induced by dietary carbohydrates. In the present study we have chosen only copper-containing indices as signs of the deficiency rather than copper-related indices, such as hemoglobin, hematocrit, and cholesterol (12) because the latter can also be affected by other dietary components.

Ceruloplasmin activity tended to be lower in copper-supplemented rats fed fructose than in rats fed starch; ceruloplasmin activity was undetectable in some animals. Similarly, lower concentrations of plasma copper and erythrocyte SOD activities were found in copper-supplemented rats fed fructose as compared to copper-supplemented rats fed starch. Previously we have not noted differences in copper status between the supplemented groups fed fructose or starch (1–6). In the present study, copper was mixed with the diets of the copper-supplemented controls. In our previous experiments (1–6), supplemented rats were given copper in their drinking water. If fructose interacts with dietary copper and interferes with its absorption, reduced copper status should be reflected also in copper-supplemented rats. Landes (13) has shown that serum copper and ceruloplasmin activity were significantly reduced by fructose feeding as compared to starch only when rats were fed excessive levels of iron. When iron was supplied in nutritionally adequate, but not excessive levels, no such difference was observed (13). In addition to reduced copper status, fructose feeding, per se, has been shown to reduce iron (2, 13) and selenium status (5) in rats of adequate copper status. The effects of fructose on iron absorption (2, 13) are contrary to those studies that reported that fructose facilitates iron absorption (14, 15).

The reasons for the decreased copper status in rats fed the fructose diet, adequate in copper, could be due to an increased requirement for copper. Greater ^{67}Cu retention in copper-supplemented rats fed fructose as compared to starch was observed when copper was administered intraperitoneally (16). Copper-supplemented rats fed fructose-containing diets had decreased insulin binding, lipogenesis, and glucose oxidation (by the isolated adipoyctes) (17), reduced insulin sensitivity (18, 19), and impaired glucose tolerance (20, 21) when compared to rats fed starch. Copper deficiency produces similar metabolic effects (17, 21, 22).

The results demonstrate that none of the measures of copper status of living animals investigated in the present study were sufficiently sensitive to demonstrate differences between degrees of severity in copper-deficient rats. On the other hand, those blood indices enabled us to differentiate between degrees of copper status in a state of copper adequacy. Although the number of animals used in each group was small ($n = 5$) and not the same rats were used each week, and although volume of blood was a limiting factor until the third week of the study, only reduced growth rate, mortality, and reduced hepatic copper levels in copper-deficient rats fed fructose, but not starch, could indicate that dietary fructose exacerbated the symptoms associated with the deficiency. Since diets consumed by subjects living in industrialized societies contain relatively high levels of fructose-containing sugars (23, 24) and inadequate amounts of copper (25), dietary fructose may lower already marginal copper levels. If degree of depletion of copper cannot be monitored by conventional methods, such as measurements of SOD, copper, and ceruloplasmin in blood, then other sensitive tests or alternative parameters to assess copper status are needed.

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