Antioxidant Defense System in Lung of Male and Female Rats: Interactions With Alcohol, Copper, and Type of Dietary Carbohydrate

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Male and female rats were used to investigate the effects of type of dietary carbohydrate (CHO), copper, and ethanol consumption on lung antioxidant enzyme activities and levels of phosphorylated compounds in whole blood. Copper-deficient female rats exhibited a greater degree of copper deficiency than males, as assessed by hepatic copper concentration and hepatic copper superoxide dismutase (CuSOD) activity. However, copper-deficient male rats fed fructose-containing diets exhibited greater growth retardation, anemia, and heart hypertrophy than females consuming the same diets and males fed starch. In addition, one of 10 copper-deficient male rats that ate a fructose-based diet and drank water and one of 10 copper-deficient male rats that ate a starch-based diet and drank ethanol died. Copper-deficient, starch-fed males exhibited the highest activities of glutathione peroxidase (GSH-Px) and catalase as compared with fructose-fed rats. Ethanol consumption elevated the activities of GSH-Px and catalase. Copper-deficient female rats exhibited higher catalase but lower GSH-Px activities than males. It is suggested that in copper deficiency, the ability to increase antioxidant enzyme activities in rats consuming starch is greater than in rats consuming fructose. Rats fed starch are provided with a greater degree of protection against oxidative damage than rats fed fructose. In addition, polyphosphorylated compounds in blood were reduced in copper-deficient male rats that consumed fructose-based diets. This may impair supply of oxygen to tissues.

COPPER IS A COMPONENT of the enzyme superoxide dismutase (CuSOD), which plays a major role in the defense against oxygen radicals derived from superoxide, hydroperoxyl, hydroxyl, and singlet oxygen by dismutating the superoxide.[1] In dietary copper deficiency, activity of this enzyme is decreased, leading to generation of oxygen radicals that are responsible for peroxidative damage to tissues.[2-6] Other factors such as the type of dietary carbohydrate (CHO) and the sex of the animal studied play a role in the expression of pathological changes in copper deficiency. When the diet contains sucrose or fructose, copper-deficient rats develop severe pathological changes and die of the deficiency.[7-9] In contrast, when the diet contains complex CHOs such as starch, pathologies are either prevented or ameliorated and rats survive.[7-9] In addition, when female and male rats consume the same copper-deficient diet containing fructose, males are more susceptible to the lethal consequences of copper deficiency than females.[10,11]

We have recently reported that only in male rats fed a fructose diet deficient in copper were free radicals detected in the liver by electron-spin resonance.[12,13] No free radicals were detected in livers of females fed the same copper-deficient diet.[13] We have also reported that livers of males fed a copper-deficient diet containing starch did not generate free radicals.[12] These results may indicate different responses of the antioxidant defense system to free radical challenge imposed by the type of dietary CHO and the gender.

Other dietary agents or deficiencies in addition to copper deficiency can be responsible for generating free radicals and tissue damage. The addition of ethanol in vitro decreases CuSOD activity.[14] It has been suggested that production of oxygen radicals during oxidation of acetaldehyde is the factor responsible for ethanol-induced liver pathology.[15,16]

We have recently reported that ethanol consumption mimicked the effects of fructose in copper deficiency.[17,18] The effects of ethanol consumption and of copper-deficient diets containing fructose on metabolic and physiological indices are remarkably similar.[17] When copper-deficient rats ate starch, they usually did not exhibit pathological changes. However, when they drank ethanol, they developed pathologies similar to those exerted by the combination of fructose feeding and copper deficiency. These changes included growth retardation, anemia, pancreatic atrophy, heart hypertrophy, and mortality.[17,18]

Copper deficiency is accompanied by changes in metabolism and properties of connective-tissue proteins such as elastin and collagen.[19] This effect is due to the role that copper plays in lysyl oxidase.[20,21] Lysyl oxidase is involved in cross-linking of collagen and elastin. It is responsible for initiation of the cross-linking process in collagen and elastin by oxidizing certain lysyl residues to aldehydes.[20,21] It is expected that nutritional copper deficiency is accompanied by degenerative changes in the tensile and elastic properties of connective tissues.[22-24]

We chose to characterize antioxidant properties of lung tissue for two reasons. First, the lung is extremely susceptible to copper deficiency. Activities of antioxidant enzymes in lung tissue are lower than in liver even in copper-adequate rats.[3,25] The alveolar epithelial surface is under constant exposure to high oxygen pressure, as well as other oxidizing substances in ambient air.[26] These agents induce oxidative stress, making the lung highly susceptible to free radical generation. For that reason, lung epithelial-lining

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fluid possesses an antioxidant activity that may be important in protecting the lung against oxidant injury.[26] In copper deficiency, this protection may be impaired.[27-31]

Second, the lung provides a good model of connective tissue. The microscopic structure of the alveolar walls consists of an extremely rich intertwining network of anastomotic capillaries that are supported by a delicate fibrous stroma enriched by elastic fibers. For these reasons, the lung of the copper-deficient rat has been proposed as a model for developmental pulmonary emphysema.[27] Morphological and physiological responses of the lung to copper deficiency and to hyperoxia have been well documented.[27-31]

However, changes in collagen and elastin are not the only contributing factors that could affect lung integrity and performance in copper deficiency. The metabolism and function of the red blood cell have shown the importance of its metabolism in contributing to the maintenance of adequate oxygen delivery. In a variety of diseases, such as lung disease and various types of anemias, an adaptive mechanism has to act to increase oxygen delivery to the tissues when less oxygen is available. In the case of certain anemias, the increased reticulocytosis will lead to a red blood cell population enriched in 2,3-diphosphoglyceric acid (2,3-DPG).[32-34] Abnormal concentrations of polyphosphate compounds such as adenosine triphosphate (ATP) and 2,3-DPG could modulate oxygen transport, which in turn could affect respiratory pulmonary physiology.[32,34]

In general, most researchers use only one type of dietary CHO and only one sex of animals. In the case of copper research, the type of dietary CHO,[6-9] ethanol,[17-18] and sex of the rat[10,11] play important roles in the expression of signs associated with copper deficiency such as anemia, heart hypertrophy, heart pathology, and mortality. The purpose of this study was to determine whether type of dietary CHO, ethanol consumption, and copper deficiency affect activities of antioxidant defense enzymes in lung tissue and levels of polyphosphate compounds in whole blood of male and female rats.

MATERIALS AND METHODS

One hundred sixty weanling Sprague-Dawley male and female rats weighing approximately 40 to 45 g each were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). All rats were housed individually in suspended stainless steel cages in quarters maintained at 20°C and 55% relative humidity with a 12-hour light/dark cycle. They were fed a copper-deficient (0.6 μg Cu/g diet) or copper-adequate (6.0 μg Cu/g) diet containing 627 g/kg CHO as either starch or fructose, with 200 g/kg egg white solids, 95 g/kg corn oil, 30 g/kg nonnutritive fiber (cellulose), 35 g/kg AIN-76 salt mix formulated in our laboratory to omit cupric carbonate,[35] and 10 g/kg AIN-76A vitamin mix[36] supplemented with 2 mg biotin and 2.7 g choline bitartrate. Copper-deficient diets contained 0.6 μg Cu/g diet as determined by atomic absorption spectrophotometry. Copper-adequate diets were prepared by adding copper carbonate to the copper-deficient mineral mixture to produce a final concentration of 6.0 μg Cu/g diet. All rats had free access either to distilled deionized drinking water or to ethanol solution. All groups had 40 rats. Half of the rats from each dietary group were trained to drink an ethanol solution beginning on the day after arrival by slowly increasing the ethanol concentration from 7% to 20% over a period of 8 days. Rats were weighed and diet and liquid intake were measured weekly. The study was terminated after 5 weeks.

Fasted rats (16 to 18 hours) were anesthetized by placing them in precharged CO_2 chambers, and blood was withdrawn from the abdominal aorta into heparinized test tubes. Aliquots of heparinized blood (1.0 mL) were added to 3.0 mL 12% trichloroacetic acid. After allowing them to stand approximately 5 minutes in an ice bath, blood samples were centrifuged at 3,000 rpm to obtain clear supernatant. Clear supernatants were used to measure both ATP and 2,3-DPG levels. The quantitative enzymatic determination of 2,3-DPG in whole blood was performed according to the method of Rose and Leibowitz.[37] Measurements of the decrease in absorbance of NADH at 340 nm served to quantify 2,3-DPG levels.[37] ATP levels were measured enzymatically in supernatants obtained from whole blood.[38] By determining the decrease in absorbance that results when NADH is oxidized to NAD, a measure of the amount of ATP originally present was obtained.[38] Blood was also collected in capillary tubes for hematocrit determination.

Approximately 1 g liver or diet was used for copper measurements. Copper levels were measured in samples by a method combining dry heat and acid digestion.[39] Duplicate samples were analyzed by flame atomic absorption spectrophotometry (Perkin-Elmer Model 5000, Norwalk, CT). National Institute of Standards and Technology (formerly National Bureau of Standards) reference material, bovine liver 1577a, was digested and analyzed along with samples to ensure accuracy. Lungs were removed, rinsed in ice-cold saline, and immediately placed in three times their volume of cold 1.15% KCl containing 0.2% Triton X-100 and homogenized. One half of the homogenate was removed for determination of glutathione peroxidase (GSH-Px) activity, and the remainder was used for determination of catalase and CuSOD. CuSOD activity in liver and lung tissues was determined following centrifugation at 700 × *g* for 10 minutes. An aliquot of the supernatant was mixed with 0.25 times the volume of ethanol and 0.15 times the volume of chloroform to remove MnSOD.[40] After centrifugation, the supernatant was assayed according to the method of Misra and Fridovich[41] using the photochemical *O*-dianisidine-riboflavin assay. GSH-Px was assayed following centrifugation of the supernatant at 105,000 × *g* by the method of Levander et al[42] as modified by Paglia and Valentine.[43] Catalase activity was measured by the method of Aebi[44] as modified by Sigma (St Louis, MO) in which the disproportionation of H_2O_2 was monitored at 240 nm.

This study was designed to examine the effect of type of dietary CHO (starch *v* fructose), level of dietary copper (deficient *v* adequate), ethanol consumption, and gender. Therefore, statistical analysis was performed using a 2 × 2 × 2 × 2 analysis of variance (ANOVA). Main effects of CHO, copper, ethanol, and gender and interactive effects were considered statistically significant at P less than .05. Pairwise comparisons of the least-square means were performed to assess significant interactions and to interpret biological importance. P less than .05 was considered statistically significant.

RESULTS

The ethanol solution was tolerated well by all rats. The ethanol group consumed as much liquid as the non-ethanol group. After 5 weeks of treatment, all copper-deficient male rats fed fructose appeared frail, growth was retarded, there was hair loss, priapism was common, and anemia was evident by the pale eyes, ears, and feet. Growth retardation and anemia were also evidenced in copper-deficient rats fed starch and given ethanol. One male copper-deficient rat that ate fructose and drank water and one male copper-deficient rat that ate starch and drank ethanol died during

the fourth week of study. There were no deaths in the other dietary groups. Rats that died had ruptured hearts in the area of the apex.

Mean body mass, relative heart and lung mass, hepatic copper concentration, and hepatic CuSOD activity are presented in Table 1. Some of these results were presented elsewhere.[45] Sex of the rat, copper, ethanol, and type of dietary CHO contributed to changes in body weight. The largest effect on body weight was exerted by gender. Females weighed less than males. Copper deficiency was the second largest contributor to depressed body weight. Ethanol and fructose followed. They both were responsible for depressed body weight. However, the effect of ethanol was of greater magnitude ($F = 83.8$ *v* 39.7 for ethanol and fructose, respectively). Fructose-fed copper-deficient rats weighed less than all other rats. Male rats fed copper-adequate diets weighed more than all other rats. Although there was a significant three-way interaction (sex × CHO × Cu), Pairwise comparison showed that the magnitude of the interaction was primarily due to sex and copper.

Relative heart mass was larger for males than for females. Copper played a major role in heart hypertrophy ($F = 205$). Copper deficiency increased relative heart mass. Fructose feeding increased relative heart size as compared with starch feeding. The combination of fructose feeding with copper deficiency resulted in a large increase of heart mass. Greater heart mass was also noted in copper-deficient males as compared with females. Relative lung size was greater in females as compared with males. Relative lung mass was further increased by starch feeding in female rats. Copper deficiency resulted in a decreased relative lung mass as compared with that of copper-adequate rats. The largest relative lung mass was found in female rats that did not consume ethanol.

Copper status was assessed by measuring hepatic copper concentration and CuSOD activity. As expected, consumption of a copper-deficient diet resulted in a reduced hepatic copper concentration as compared with levels in copper-adequate controls in both male and female rats ($F = 1146$). Females exhibited a lower concentration of hepatic copper than males ($F = 10.69$). Ethanol consumption reduced hepatic copper ($F = 18.73$). The combination of copper deficiency and ethanol consumption resulted in the lowest

Table 1. Body Mass, Relative Heart and Lung Mass, Hepatic Copper Concentration, and CuSOD Activity

Parameter	Body Mass (g)	Relative Heart Mass (g/100 g BW)	Relative Lung Mass (g/100 g BW)	Hepatic Copper (μg/g WW)	Hepatic CuSOD (U/mg protein)
Males					
FR − Cu	156 ± 6	0.81 ± 0.06	0.65 ± 0.03	1.20 ± 0.23	4.11 ± 0.99
FR − Cu + E	147 ± 6	0.79 ± 0.04	0.72 ± 0.02	0.87 ± 0.08	3.22 ± 0.31
ST − Cu	217 ± 8	0.54 ± 0.06	0.57 ± 0.02	1.56 ± 0.33	4.12 ± 0.60
ST − Cu + E	172 ± 5	0.66 ± 0.04	0.63 ± 0.03	1.11 ± 0.11	4.68 ± 0.36
FR + Cu	225 ± 3	0.40 ± 0.02	0.69 ± 0.04	4.27 ± 0.11	10.43 ± 0.38
FR + Cu + E	201 ± 2	0.43 ± 0.01	0.70 ± 0.03	3.52 ± 0.17	10.32 ± 0.41
ST + Cu	238 ± 5	0.40 ± 0.01	0.66 ± 0.02	4.28 ± 0.29	11.15 ± 1.53
ST + Cu + E	190 ± 6	0.44 ± 0.01	0.68 ± 0.02	3.88 ± 0.21	8.34 ± 0.82
Females					
FR − Cu	135 ± 4	0.62 ± 0.02	0.73 ± 0.01	0.72 ± 0.09	3.02 ± 0.38
FR − Cu + E	124 ± 3	0.62 ± 0.03	0.68 ± 0.01	0.59 ± 0.03	2.85 ± 0.47
ST − Cu	160 ± 3	0.53 ± 0.01	0.77 ± 0.05	1.00 ± 0.13	3.21 ± 0.61
ST − Cu + E	136 ± 4	0.49 ± 0.01	0.71 ± 0.02	1.02 ± 0.12	3.91 ± 0.79
FR + Cu	149 ± 3	0.43 ± 0.01	0.79 ± 0.04	4.23 ± 0.15	14.00 ± 1.24
FR + Cu + E	149 ± 3	0.44 ± 0.02	0.76 ± 0.02	3.55 ± 0.12	13.17 ± 2.27
ST + Cu	155 ± 3	0.41 ± 0.01	0.86 ± 0.06	3.92 ± 0.11	14.73 ± 1.84
ST + Cu + E	142 ± 3	0.44 ± 0.01	0.81 ± 0.06	3.48 ± 0.14	13.41 ± 1.64
ANOVA (*P*)					
Sex	.0001	.0001	.0001	.0014	.0047
CHO	.0001	.0001	NS	NS	NS
Sex × CHO	.0039	NS	.0030	NS	NS
Cu	.0001	.0001	.0004	.0001	.0001
Sex × Cu	.0001	.0001	NS	NS	.0001
CHO × Cu	.0001	.0001	NS	.0473	NS
Sex × CHO × Cu	.0141	NS	NS	NS	NS
E	.0001	NS	NS	.0001	NS
Sex × E	.0001	NS	.0126	NS	NS
CHO × E	.0001	NS	NS	NS	NS
Sex × CHO × E	NS	NS	NS	NS	NS
Cu × E	.0354	NS	NS	.0368	NS
CHO × Cu × E	NS	NS	NS	NS	NS
Sex × CHO × Cu × E	NS	NS	NS	NS	NS

NOTE. Results are the mean ± SEM of 10 observations per group, except for 9 FR − Cu males and 9 ST − Cu + E males.
Abbreviations: FR, fructose; ST, starch; E, ethanol; NS, not significant; BW, body weight; WW, wet weight.

concentration of hepatic copper. Fructose feeding further reduced hepatic copper in comparison to starch feeding. Males had higher CuSOD activity than females when they consumed a copper-deficient diet, but females had higher activity when they consumed a copper-adequate diet. Copper played a major role in inducing the activity of CuSOD. Copper deficiency reduced hepatic CuSOD activity in all rats regardless of gender, dietary CHO, or ethanol as compared with copper-adequate rats.

ATP concentration was higher in females than in males and in starch feeding than in fructose feeding (Table 2). Copper deficiency also reduced whole-blood ATP. The lowest concentration of ATP was found in copper-deficient males. A similarly low concentration of ATP was found in copper-deficient rats fed fructose. Ethanol increased ATP concentration. Although there was a significant sex × CHO × Cu interaction, the contribution of gender was of minor magnitude as compared with CHO and copper.

The whole-blood concentration of 2,3-DPG was higher in females than in males (Table 2). It was lower in fructose-fed than in starch-fed rats. Whole-blood 2,3-DPG was also reduced by copper deficiency. The concentration of 2,3-DPG was lower in copper-deficient males fed fructose in comparison to females and to rats fed starch. The significant interactions affecting 2,3-DPG were mainly due to copper (F = 53); sex and type of dietary CHO had smaller effects (F = 9.46 and 7.72, respectively). Ethanol had no effect on 2,3-DPG.

Table 2. ATP and 2,3-DPG in Whole Blood and Hematocrit

Parameter	ATP (μmol/dL)	2,3-DPG (μmol/mL)	Hematocrit (%)
Males			
FR − Cu	27.0 ± 3.5	1.41 ± 0.15	25.6 ± 4.8
FR − Cu + E	30.6 ± 2.3	1.26 ± 0.16	19.1 ± 2.4
ST − Cu	54.7 ± 2.5	2.57 ± 0.13	36.1 ± 2.2
ST − Cu + E	53.5 ± 1.6	2.26 ± 0.09	29.3 ± 4.5
FR + Cu	63.0 ± 6.9	3.21 ± 0.10	44.6 ± 1.9
FR + Cu + E	62.5 ± 1.7	2.86 ± 0.08	44.1 ± 1.6
ST + Cu	64.1 ± 0.9	2.93 ± 0.06	46.7 ± 1.9
ST + Cu + E	66.6 ± 2.7	2.88 ± 0.08	43.7 ± 1.4
Females			
FR − Cu	41.1 ± 2.6	2.54 ± 0.20	27.4 ± 1.1
FR − Cu + E	41.3 ± 1.2	2.52 ± 0.42	29.3 ± 1.4
ST − Cu	58.1 ± 2.4	2.65 ± 0.11	43.8 ± 0.9
ST − Cu + E	67.5 ± 7.1	2.66 ± 0.17	40.4 ± 1.5
FR + Cu	48.8 ± 4.6	2.82 ± 0.15	44.2 ± 0.6
FR + Cu + E	61.5 ± 2.9	2.97 ± 0.09	44.7 ± 0.6
ST + Cu	70.2 ± 2.6	3.06 ± 0.13	47.6 ± 0.7
ST + Cu + E	72.1 ± 3.9	2.79 ± 0.22	46.7 ± 0.7
ANOVA (*P*)			
Sex	.0086	.0004	.0001
CHO	.0001	.0025	.0001
Sex × CHO	NS	.0276	NS
Cu	.0001	.0001	.0001
Sex × Cu	.0020	.0004	.0013
CHO × Cu	.0001	.0023	.0001
Sex × CHO × Cu	.0189	.0041	NS
E	.0477	NS	NS
Sex × E	NS	NS	NS
CHO × E	NS	NS	NS
Sex × CHO × E	NS	NS	.0322
Cu × E	NS	NS	NS
Sex × Cu × E	NS	NS	NS
CHO × Cu × E	NS	NS	NS
Sex × CHO × Cu × E	NS	NS	.0158

NOTE. Abbreviations as in Table 1.

Females exhibited higher hematocrits than males (F = 13.82). Fructose feeding reduced hematocrits as compared with starch feeding (F = 36.72). Copper deficiency reduced hematocrits (F = 165.13). The combination of fructose feeding and copper deficiency resulted in the lowest hematocrits. Similarly, copper-deficient male rats exhibited the lowest hematocrits. Ethanol consumption by itself had no effect on hematocrit (F = 0.42). However, the combination of ethanol and fructose in the male (sex × CHO × Cu) became significant (F = 4.93). Similarly, the combination of sex × CHO × Cu was significant (F = 3.94), with ethanol having the least significant effect.

The activity of CuSOD in lung tissue was greatly affected by gender. Females exhibited lower activity than males. Starch-fed rats had higher activity than fructose-fed rats. Males that consumed starch had higher activity than males fed fructose or females fed starch and fructose. Copper deficiency reduced CuSOD activity as compared with levels in copper-adequate controls in females but not in males. GSH-Px activity of lung was lower in females than in males (Table 3). It was higher in starch-fed rats than in fructose-fed rats. Copper-deficient male rats had elevated GSH-Px activity as compared with copper-adequate rats and females. The activity of GSH-Px was also increased in copper-deficient rats by starch feeding. Ethanol consumption elevated GSH-Px in all rats; however, starch feeding further increased its activity. The highest GSH-Px activity was found in starch-fed, copper-deficient male rats.

Rats fed starch had elevated catalase activity as compared with rats fed fructose. Copper-deficient animals had reduced catalase activity as compared with copper-adequate animals. Copper deficiency combined with fructose feeding caused a reduction in catalase activity. Ethanol elevated catalase activity in all animals. Female rats that consumed a copper-adequate diet and drank ethanol had the highest catalase activity.

DISCUSSION

As previously reported, type of dietary CHO,[6-9] sex of the rat,[10,11] ethanol consumption,[17,18] and copper intake played a role in the signs associated with copper deficiency. Fructose feeding in males aggravated the signs associated with copper deficiency, and one rat died prematurely. In agreement with previous studies,[17,18] ethanol consumption mimicked fructose-feeding effects in copper-deficient male rats fed starch. Ethanol consumption by males fed a copper-deficient diet containing starch caused a reduction in body weight, increased heart size, and anemia as compared with copper-deficient rats that ate starch and drank water. In addition, one rat died of heart-related abnormalities.

As previously reported,[10,11] signs associated with copper deficiency in females fed fructose were less severe than those found in males fed the same diet. However, hepatic

Table 3. Cu-SOD, GSH-Px, and Catalase Activities in Lung Tissue

Parameter	CuSOD*	GSH-Px†	Catalase‡
Males			
FR − Cu	3.28 ± 0.29	3.52 ± 0.12	9.48 ± 1.46
FR − Cu + E	3.39 ± 0.40	3.86 ± 0.05	12.59 ± 1.55
ST − Cu	3.96 ± 0.28	4.09 ± 0.10	18.00 ± 1.19
ST − Cu + E	4.16 ± 0.171	4.18 ± 0.07	18.39 ± 0.83
FR + Cu	3.17 ± 0.25	3.44 ± 0.09	17.24 ± 0.87
FR + Cu + E	3.08 ± 0.29	3.53 ± 0.06	21.83 ± 2.01
ST + Cu	3.51 ± 0.29	3.45 ± 0.10	17.42 ± 1.13
ST + Cu + E	3.21 ± 0.37	3.45 ± 0.08	21.01 ± 2.02
Females			
FR − Cu	2.23 ± 0.34	3.44 ± 0.11	13.48 ± 0.70
FR − Cu + E	0.98 ± 0.41	3.78 ± 0.09	14.88 ± 1.20
ST − Cu	2.16 ± 0.53	3.67 ± 0.13	19.60 ± 1.75
ST − Cu + E	1.82 ± 0.18	3.68 ± 0.10	20.57 ± 1.64
FR + Cu	3.29 ± 0.29	3.14 ± 0.08	16.58 ± 2.63
FR + Cu + E	2.61 ± 0.18	3.28 ± 0.05	19.94 ± 1.07
ST + Cu	3.32 ± 0.18	3.12 ± 0.12	19.50 ± 1.49
ST + Cu + E	3.36 ± 0.07	3.35 ± 0.05	25.11 ± 5.24
ANOVA (*P*)			
Sex	.0001	.0001	NS
CHO	.0151	.0135	.0001
Sex × CHO	.0001	NS	NS
Cu	.0124	.0001	.0001
Sex × Cu	.0001	.0001	NS
CHO × Cu	NS	.0066	.0204
Sex × CHO × Cu	NS	.0210	NS
E	NS	.0021	.0093
Sex × E	NS	.034	NS
CHO × E	NS	NS	NS
Sex × CHO × E	NS	NS	NS
Cu × E	NS	NS	NS
Sex × Cu × E	NS	NS	.0438
CHO × Cu × E	NS	NS	NS
Sex × CHO × Cu × E	NS	NS	NS

NOTE. Abbreviations as in Table 1.

*U/mg protein.

†μmol NADPH oxidized/min/mg protein.

‡μmol H_2O_2 decomposed by disproportionation/min/mg protein.

copper concentration was lower in females than in males. This low copper concentration in livers of females was correlated with low activity of CuSOD. Since CuSOD is the enzyme that forms the first line of defense against oxygen radicals[1] derived from superoxide, it was expected that copper-deficient female rats should be more susceptible to peroxidative damage due to lack of defense against free radical generation. In addition, GSH-Px activity was lower in females than in males. However, we reported previously that free radicals were generated only in copper-deficient males that consumed fructose, not in females fed fructose.[13] This finding may indicate that compensatory mechanisms such as MnSOD play a role in protecting cellular components against free radical–induced pathways in the female.[46] On the other hand, differences in metabolic pathways of fructose between male and female rats could play a role.[47]

The lung is an organ sensitive to changes in copper nutriment, since its structural integrity is dependent on cross-linking of elastin brought about by the copper-containing enzyme, lysyl oxidase. Lung function can be affected by different types of collagen and elastin. Steroid hormones, particularly estrogens, affect connective-tissue metabolism,[48,49] and the maturation process of elastin is increased after estrogen administration.[48] Activity of lysyl oxidase is higher in females than in males. Insoluble collagen is also increased in females as compared with males. Reduced activity of lysyl oxidase in the male rat is correlated with an increase of soluble collagen in heart tissue.[50,51] However, consumption of fructose-based diets in copper-deficient rats caused a greater reduction of lysyl oxidase activity than consumption of starch-based diets. This fructose effect resulted in a greater amount of soluble collagen as compared with starch feeding (M. Werman and S. Bhathena, personal communication, April 1994).

It is not clear why ethanol consumption increased lung mass in males only. Ethanol had no effect on collagen levels in the rat when it was administered in drinking water.[52] However, it did have an effect on collagen synthesis and maturation when it was given as an integral part of a liquid diet and for a long period.[53] Unidentified metabolites of ethanol may affect connective-tissue integrity.[54-56]

Because of its role as a portal route for inhaled compounds and its high blood flow in relation to other organs, the lung is an important organ in terms of exposure to toxic substances. Indeed, the lung is also under constant oxidative stress, since it has immediate susceptibility to activated-oxygen insult. Copper-deficient animals are more susceptible to lung damage than controls when they are exposed to a hyperoxic environment.[28,29] This damage is structural[27] and functional[28,29] in nature.

The objectives of this study were to measure lung tissue activities of three enzymes participating in the free radical defense system in rats consuming different levels of dietary copper, different types of dietary CHO, and ethanol. In general, in the present study CuSOD activity in lung tissue was not greatly affected by dietary copper deprivation. It was higher in copper-deficient than in copper-adequate males. This finding is intriguing, since CuSOD activity in the liver of all rats was highly sensitive to copper deficiency regardless of dietary CHO or sex (Table 1). CuSOD activity is sensitive to exposure to hyperoxia.[28] The depressed CuSOD activity in the lung of copper-adequate rats could be due to exposure of the rats to a precharged CO_2 chamber. We do not have data to support this hypothesis. However, carbon dioxide has been shown to affect secretion of enzymes and/or hormones from tissues.[57] The data pertaining to lung SOD may not reflect normal physiological conditions.

In the present study, GSH-Px was significantly higher in males than in females when the diet was inadequate in copper and ethanol was consumed. The highest activity was noted in copper-deficient male rats fed starch. However, the magnitude of these differences between dietary groups was small. The effect of copper deficiency on GSH-Px was tissue-specific. GSH-Px activity of lung tissue is lower than that of liver[3,25] and is not as sensitive to dietary copper deprivation as GSH-Px of liver.[25,27-29,58] We have previously shown that fructose feeding per se decreased GSH-Px activity in the liver as compared with starch feeding.[6]

Copper deficiency further reduced the activity of GSH-Px, although all animals in that study ate a selenium-adequate diet.[6] Dietary copper intake may affect selenoenzyme metabolism by influencing mRNA stability or processing of the primary transcript to the mature mRNA.[59] However, these effects of copper on selenium enzymes were manifested when the copper-deficient diet contained sucrose.[58,59] There are no reports of the effect of copper deficiency on mRNA of GSH-Px in starch-fed rats.

Catalase activity was depressed by copper deficiency. It was further decreased by fructose feeding. Since catalase is a heme protein, impaired heme synthesis could result in decreased catalase activity. Impaired heme synthesis reflected by decreased hematocrits was noted in copper-deficient rats fed fructose. However, ethanol consumption increased catalase activity. An ethanol-induced increase in catalase activity is not surprising, since one of the pathways of ethanol metabolism is associated with peroxisomal catalase.[60]

In recent years, several biphosphorylated compounds, including 2,3-DPG, have been recognized as regulatory molecules implicated in the control of metabolism and other cellular functions.[33] The major role of 2,3-DPG in erythrocytes is to act as an allosteric modulator of the affinity of hemoglobin for oxygen.[32-34] 2,3-DPG bonds preferentially with deoxyhemoglobin and displaces the $HbO_2 \rightleftarrows Hb$ equilibrium to the deoxygenated form. This facilitates the supply of oxygen to tissues. In the present study, 2,3-DPG was significantly reduced by copper deficiency and fructose feeding in the male rat. Low levels of 2,3-DPG should cause a lower oxygen affinity in erythrocytes, which in turn may increase CO_2 bonding. Alterations in oxygen affinity in red blood cells should affect other cell functions. This is the first demonstration that copper deficiency, fructose feeding, and gender affect 2,3-DPG concentration in whole blood. Reasons for these effects in copper-deficient male rats fed fructose are not fully understood. Changes in 2,3-DPG concentration in whole blood have been reported due to alterations in metabolic or physiological conditions.[32-34] Fructose and copper deficiency[6,61] induce perturbations in hepatic phosphorylated metabolites.[62-65] Decreases in hepatic P_i and ATP occur after a load of fructose[62-65] and in copper deficiency.[6,8] Enzymatic reactions implicated in the synthesis or breakdown of 2,3-DPG could also be responsible for the results reported here. Lactic acidosis is a common feature in rats fed fructose,[65] and it could also play a role.[62] The presence of severe anemia in copper-deficient male rats fed fructose will magnify the impaired supply of oxygen to tissues. The anemia of copper deficiency was of similar magnitude in females and males when the diet contained fructose. However, 2,3-DPG and ATP were depressed only in males. It is generally accepted that red blood cell 2,3-DPG levels are inversely related to anemia.[32-34] This is an adaptive mechanism that acts to increase oxygen delivery to tissues when less oxygen is available.[32-34] This mechanism does not seem to operate in the copper-deficient male rat that consumes a fructose-based diet.

Depletion of ATP following administration of fructose has been documented in male rats.[62] Similarly, a reduction of ATP has been reported to occur in copper-deficient male rats.[6,8,61] In the present study, feeding of a fructose diet deficient in copper to male but not to female rats resulted in an approximate 50% reduction of ATP. Reasons for the gender effect on ATP synthesis are not fully understood. However, differences in metabolic pathways of fructose in male and female rats[47] can be responsible for the depletion of adenine nucleotides in the male. In erythrocytes, ATP, like 2,3-DPG, profoundly decreases the oxygen affinity of hemoglobin.[32] As with 2,3-DPG, a decrease of ATP in copper-deficient male rats fed fructose could have severe consequences due to the impaired supply of oxygen to tissues. The combination of reductions of both polyphosphate compounds (2,3-DPG and ATP) that regulate oxygen affinity of red blood cells could result in an oxygen deficit, which in turn could affect respiratory physiology.

Diets consumed by subjects living in industrialized societies, including the United States, contain relatively high levels of sugars such as sucrose and fructose[66-67] and marginal amounts of copper.[68] With the introduction of high-fructose corn sweeteners in 1970, the amount of fructose in the US food supply has increased.[66,67] The use of high-fructose corn sweeteners has doubled from 13 lb per capita per year in 1977 to 26 lb per capita per year in 1980.[66] In 1984, the annual per capita intake in terms of absolute alcohol was 2.65 gal per person 14 years of age or older.[69,70] In addition, the prevalence of alcoholism in general hospitals was 18% for men and 5.5% for women.[70] Since one third of the US adult population abstains from alcohol, those who drink consume an average of 1.3 oz absolute alcohol per day. If the same type of interaction between dietary CHO, ethanol, and copper status found in rats also occurs in humans, then the high intake of fructose and/or ethanol may affect already marginal copper levels due to inadequate copper intake.

In conclusion, results from this experiment indicate that variations in dietary CHO, copper level, gender, and ethanol consumption affect the signs associated with copper deficiency, the antioxidant enzyme system of lung tissue, and the supply of oxygen to tissues. However, the magnitude of the effects was exerted mainly by copper, gender, and type of dietary CHO. Ethanol played a minor role either by itself or as part of an interaction. The combination of inadequate dietary copper intake with fructose consumption resulted in a depressed ability of the lung to protect itself against free radical insult.

REFERENCES

1. McCord JM, Fridovich I: Superoxide dismutase: An enzymatic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049-6055, 1969

2. Taylor CG, Bettger WJ, Bray TM: Effect of dietary zinc or copper deficiency on the primary free radical defense system in rat. J Nutr 118:613-621, 1988

3. Paynter DI, Moir RJ, Underwood EJ: Changes in activity of Cu-Zn superoxide dismutase enzyme in tissues of the rat with changes in dietary copper. J Nutr 109:1570-1576, 1979

4. Balevska PS, Russanov E, Kassabova TA: Studies on lipid peroxidation in rat liver by copper deficiency. Int J Biochem 12:489-493, 1981

5. Davis NT, Sarkozy P: The effects of copper deficiency on lipid peroxidation in rat liver microsomes, in Mills CF, Brenner I, Chesters JK (eds): Trace Elements in Man and Animals. Slough, UK, Commonwealth Agricultural Bureaux, 1985, pp 39-42

6. Fields M, Ferretti RJ, Smith JC, et al: Effects of the interaction of type of dietary carbohydrate with copper deficiency on lipid peroxidation in rat tissues. Biol Trace Elem Res 6:379-391, 1984

7. Fields M, Ferretti RJ, Smith JC, et al: The effect of copper deficiency on metabolism and mortality in rats fed sucrose or starch diets. J Nutr 113:1335-1345, 1983

8. Reiser S, Ferretti RJ, Fields M, et al: Role of dietary fructose in the enhancement of mortality and biochemical changes associated with copper deficiency in rats. Am J Clin Nutr 38:214-222, 1983

9. Redman RS, Fields M, Reiser S, et al: Dietary fructose exacerbates the cardiac abnormalities of copper deficiency in rats. Atherosclerosis 74:203-214, 1988

10. Fields M, Lewis CG, Scholfield DJ, et al: Female rats are protected against the fructose induced mortality of copper deficiency. Proc Soc Exp Biol Med 183:145-149, 1986

11. Fields M, Lewis CG, Beal T, et al: Sexual differences in the expression of copper deficiency in rats. Proc Soc Exp Biol Med 186:183-187, 1987

12. Fields M, Lewis CG, Lure MD, et al: The severity of copper deficiency can be ameliorated by deferoxamine. Metabolism 40:105-109, 1991

13. Fields M, Lewis CG, Lure MD, et al: The influence of gender on developing copper deficiency and on free radical generation of rats fed a fructose diet. Metabolism 41:989-994, 1992

14. Ledig M, M'Paria JR, Louis JC, et al: Effect of ethanol on superoxide dismutase activity in cultured neural cells. Neurochem Res 5:1155-1162, 1980

15. Oei HH, Stroo WE, Burton KP, et al: A possible role of xanthine oxidase in producing oxidative stress in the heart of chronically treated rats. Res Commun Chem Pathol Pharmacol 38:453-461, 1982

16. Fridovich I: Oxygen radicals from acetaldehyde. Free Rad Biol Med 7:557-558, 1989

17. Fields M, Lewis CG: Alcohol consumption aggravates copper deficiency. Metabolism 39:610-613, 1990

18. Fields M, Lewis CG, Lure MD: Alcohol consumption mimics the effects of a high-fructose, low-copper diet in rats. Alcohol 11:17-23, 1994

19. Radhakushnamurthy B, Rucker R, Berenson G: Composition of glycosaminoglycans in the lungs of copper-deficient chicks. Proc Soc Exp Biol Med 180:392-399, 1985

20. Siegel RC, Martin GR: Collagen-crosslinking. Enzymatic synthesis of lysine-derived aldehydes and the production of crosslinked components. J Biol Chem 245:1653-1658, 1970

21. Sandberg LB, Davidson JM: Elastin and its genes, in Hearn MTW (ed): Peptides and Protein Reviews, vol 3. New York, NY, Dekker, 1984, pp 169-226

22. Hill KE, Davidson JM: Induction of increased collagen and elastin biosynthesis in copper-deficient pig aorta. Atherosclerosis 6:98-104, 1986

23. Weissman N, Shields G, Carnes W: Cardiovascular studies on copper-deficient swine. IV. Content and solubility of the aortic elastin, collagen and hexosamine. J Biol Chem 238:3115-3118, 1963

24. Buckingham K, Heng-Khoo CS, Dublick M: Copper deficiency and elastin metabolism in avian lung. Proc Soc Exp Biol Med 166:310-319, 1981

25. Jenkinson SG, Lawrence RA, Burk RF, et al: Effect of copper deficiency on the activity of the selenoenzyme glutathione peroxidase and on excretion and tissue retention of $^{75}SeO_3^{2-}$. J Nutr 112:197-204, 1982

26. Pacht ER, Davis WB: Role of transferrin and ceruloplasmin in antioxidant activity of lung epithelial lining fluid. J Appl Physiol 64:2092-2099, 1988

27. O'Dell BL, Kilburn KH, Wayland MD, et al: The lung of the copper-deficient rat. A model for developmental preliminary emphysema. Am J Pathol 91:413-432, 1978

28. Taylor CG, Bray TM: Increased lung copper-zinc superoxide dismutase activity and absence of magnetic resonance imaging–detectable lung damage in copper-deficient rats exposed to hyperoxia. J Nutr 121:467-473, 1991

29. Spence TH, Jenkinson SG, Johnson KH, et al: Effects of bacterial endotoxin on protecting copper-deficient rats from hyperoxia. J Appl Physiol 61:982-987, 1986

30. Jenkinson SG, Lawrence RA, Grafton WD, et al: Enhanced pulmonary toxicity in copper-deficient rats exposed to hyperoxia. Fund Appl Toxicol 4:170-177, 1984

31. Hammermueller JD, Bray TM, Bettger WJ: Effect of zinc and copper deficiency on microsomal NADPH-dependent active oxygen generation in rat lung and liver. J Nutr 117:894-901, 1987

32. Benesch R, Benesch RE: Intracellular organic phosphates as regulators of oxygen release by hemoglobin. Nature 221:618-622, 1969

33. Carreras J, Bartrons R, Climent F, et al: Biophosphorylated metabolites of glycerate, glucose and fructose: Functions, metabolism and molecular pathology. Clin Biochem 19:348-358, 1986

34. Brawer GJ, Eaton JW: Erythrocyte metabolism: Interaction with oxygen transport. Science 171:1205-1211, 1971

35. Anonymous: American Institute of Nutrition. Report of the AIN ad hoc committee on standards for nutritional studies. J Nutr 107:1340-1348, 1977

36. Anonymous: American Institute of Nutrition. Second report of the AIN ad hoc committee on standards for nutritional studies. J Nutr 110:1726, 1980

37. Rose ZB, Leibowitz J: Direct determination of 2,3-diphosphoglycerate. Anal Biochem 35:177-182, 1970

38. Adams H: Adenosine-5′-triphosphate determination with phosphoglycerate kinase, in Bergmeyer HU (ed): Methods of Enzymatic Analysis. New York, NY, Academic, 1963, pp 539-543

39. Hill AD, Patterson KY, Veillon C, et al: Digestion of biological materials for mineral analysis using a combination of wet and dry ashing. Anal Chem 58:2340-2342, 1986

40. Paynter DI: Changes in activity of the manganese superoxide dismutase enzyme in tissues of the rat with changes in dietary manganese. J Nutr 110:437-447, 1980

41. Misra HP, Fridovich I: Superoxide dismutase: Photochemical augmentation assay. Arch Biochem Biophys 181:308-312, 1977

42. Levander OA, DeLoach DP, Morris VC, et al: Platelet glutathione peroxidase activity as an index of selenium status in rats. J Nutr 113:55-63, 1983

43. Paglia DE, Valentine WN: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158-168, 1979

44. Aebi H: Catalase in vitro. Methods Enzymol 105:121-126, 1984

45. Fields M, Lure MD, Lewis CG: The effect of alcohol consumption in male and female rats on antioxidant defense systems: Interactions with copper status and dietary carbohydrate, in Collery PH, Poirier LA, Littlefield NA, et al: Metal Ions in Biology and Medicine, vol 3. Paris, France, Libbey Eurotex, 1994, pp 467-472

46. Rosenbaum JS, Zindenberg-Cherr S, Keen CL: Influence of copper status on the response to acute ethanol exposure in rats. Alcohol 8:473-479, 1991

47. Fields M, Lewis CG, Beal T: Accumulation of sorbitol in copper deficiency: Dependency on gender and type of dietary carbohydrate. Metabolism 38:371-375, 1989

48. Sanada H, Shikato J, Hamamoto H, et al: Changes in collagen cross-linking and lysyl oxidase by estrogen. Biochim Biophys Acta 541:408-413, 1978

49. Shoshan S, Finkelstein S: Lysyl oxidase: A pituitary hormone-dependent enzyme. Biochim Biophys Acta 439:358-362, 1976

50. Owens CA: Copper proteins and other compounds, in Owens CA (ed): Biochemical Aspects of Copper. Park Ridge, NJ, Noyes, 1982, pp 1-127

51. Coulson WF, Carnes WH: Cardiovascular studies on copper-deficient swine. IX. Repair of vascular defects in deficient swine treated with copper. Am J Pathol 50:861-868, 1967

52. Rayssiguier Y, Chavalier F, Bonnet M, et al: Influence of magnesium deficiency on liver collagen after carbon tetrachloride or ethanol administration to rats. J Nutr 115:1656-1662, 1985

53. Lieber CS: Metabolism and metabolic effects of alcohol. Semin Hematol 17:85-95, 1980

54. Feinman L, Lieber CS: Hepatic collagen metabolism: Effect of alcohol consumption in rats and baboons. Science 176:195-199, 1972

55. Laposata E, Lange LG: Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. Science 231:497-499, 1986

56. Zagorul'ko AK, Fisik EE, Tikus EN: Effect of alcohol poisoning during pregnancy on the lung surfactant systems of newborn rats. Bull Exp Biol Med 110:1031-1034, 1990

57. Bhathena SJ: Comparison of effects of decapitation and anesthesia on metabolic and hormonal parameters in Sprague-Dawley rats. Life Sci 50:1649-1655, 1992

58. Olin KL, Walter RM, Keen CL: Copper deficiency affects selenoglutathione peroxidase and selenodeiodinase activities and antioxidant defense in weanling rats. Am J Clin Nutr 59:654-658, 1994

59. Prohaska JR, Sunde RA, Zinn KR: Livers from copper-deficient rats have lower glutathione peroxidase activity and mRNA levels but normal liver selenium. J Nutr Biochem 3:429-436, 1992

60. Lieber CS, Lasker JM, Alderman J, et al: The microsomal ethanol oxidizing system and its interaction with other drugs, carcinogens and vitamins. Ann NY Acad Sci 492:11-24, 1987

61. Kopp SJ, Klevay LM, Feliksik JM: Physiological and metabolic characterization of cardiomyopathy induced by chronic copper deficiency. Am J Physiol 245:H855-H866, 1983

62. Van Den Berghe G, Bronfman M, Venneste R, et al: The mechanism of adenosine triphosphate depletion in the liver after a load of fructose. Biochem J 162:601-609, 1977

63. Van den Berghe G: Fructose: Metabolism and short-term effects on carbohydrate and purine metabolic pathways. Prog Biochem Pharmacol 21:1-32, 1986

64. Masson S, Henriksen O, Stengaard A, et al: Hepatic metabolism during constant infusion of fructose; comparative studies with ^{31}P-magnetic resonance spectroscopy in man and rats. Biochim Biophys Acta 1199:166-174, 1994

65. Van den Berghe G: Metabolic effects of fructose in the liver. Curr Top Cell Regul 13:97-125, 1978

66. Woteki CE, Welsh SO, Raper W, et al: Recent trends and levels of dietary sugars and other caloric sweeteners, in Reiser S (ed): Metabolic Effects of Utilizable Carbohydrates. New York, NY, Dekker, 1982, pp 1-27

67. US Department of Agriculture: Sugar and Sweetener. Situation and Outlook Report. US Department of Agriculture, Economic Research Service, SSSV19N2, 1994

68. Klevay LM, Viestenz KE: Abnormal electrocardiograms in rats deficient in copper. Am J Physiol 240:H185-H189, 1981

69. US Department of Health and Human Services: Sixth Special Report to the U.S. Congress on Alcohol and Health From the Secretary of Health and Human Services. DHHS Publication No. (ADM) 87-1519. Washington, DC, US Government Printing Office, 1987

70. US Department of Health and Human Services: The Surgeon General's Report on Nutrition and Health. DHHS Publication No. (PHS) 88-50210. Washington, DC, US Government Printing Office, 1988