PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **186**, 183–187 (1987)

Sexual Differences in the Expression of Copper Deficiency in Rats (42600)

MEIRA FIELDS,[1] CHARLES G. LEWIS, TODD BEAL, DANIEL SCHOLFIELD, KRISTINE PATTERSON, JAMES C. SMITH, AND SHELDON REISER

Division of Endocrinology, Department of Medicine, Georgetown University Medical School, Washington, D.C. 20007, and Carbohydrate and Vitamin and Mineral Nutrition Laboratories, USDA, BHNRC, Beltsville, Maryland 20705

Abstract. The present investigation was undertaken to establish whether the severity of copper deficiency in rats fed diets containing fructose is affected by the presence and type of endogenous sex hormones. Intact and castrated male rats and intact and ovariectomized females were fed from weaning a copper-deficient diet (0.6 ppm) containing 62% fructose for 8 weeks. Regardless of castration, male rats were anemic, exhibited heart hypertrophy, and died of the deficiency. However, castration ameliorated the anemia and delayed the mortality. In contrast, none of the females died of the deficiency. It is suggested that in addition to the sex of the animal, levels of testosterone in the male may also play a role in the severity of copper deficiency.

We have recently shown that although male and female rats fed a copper-deficient diet containing fructose are equally copper deficient (1), only male rats are anemic, have hypertrophied hearts, and die of the deficiency (1). In contrast, copper-deficient female rats are not anemic, they do not exhibit heart hypertrophy and pathology, and they survive. Thus, female rats are protected against the mortality of copper deficiency (1). It is possible that this protection is provided by the presence of endogenous estrogens. Endogenous as well as exogenous estrogens have been shown to alter the subcellular distribution of copper in the liver (2, 3) and increase plasma copper levels by inducing the synthesis of ceruloplasmin (4). Conversely, the presence of testosterone could predispose the male rats to the lethal effect of the copper–fructose interaction. If estrogens protect but testosterone exacerbates the symptoms associated with copper deficiency, then ovariectomized females should be susceptible but castrated males should be protected against the lethal effects of copper deficiency.

Thus, the purpose of the present investigation was to verify whether the expression and the severity of copper deficiency in male and female rats fed a fructose-containing diet are affected by the presence of endogenous sex hormones.

Materials and Methods. Intact male (n = 37), castrated male (n = 12), intact female (n = 12), and ovariectomized female (n = 12) Sprague–Dawley rats (Harlane–Sprague–Dawley, Indianapolis, IN) weighing approximately 45–55 g each were fed from weaning a copper-deficient (0.6 ppm) diet containing 62% fructose. The composition of the diet has been described elsewhere (5). The male rats were anesthetized with ether and the testes were removed. All intact animals were sham operated. During the fourth and eighth week of the study rats were bled from the tip of their tail and blood was collected into heparinized capillary tubes for hematocrit determination. At the end of the eighth week, six rats from each group were decapitated, and blood was collected into heparinized test tubes for the analysis of copper and ceruloplasmin activity (6) and for the determination of total estrogens and testosterone by a radioimmunoassay (7). Superoxide dismutase (SOD) activity was measured in erythrocytes (8). Livers were removed for the analysis of hepatic copper concentration. Tissues and diets were digested for mineral determinations by combining dry heat and acid digestion (9). To assure accuracy, National Bureau of Standards certified reference materials were digested and analyzed along with

[1] To whom all reprint requests should be addressed at USDA, ARS, BHNRC, Vitamin and Mineral Nutrition Laboratory, Building 307, Room 117, BARC-East, Beltsville, MD 20705.

0037-9727/87 $1.50

tissues and diets. Concentrations of copper in plasma, liver, and diets were measured by atomic absorption spectroscopy (10). Data were analyzed by ANOVA using the SAS software system for data analysis (11). A probability level of 5% was used to detect statistical significance. Duncan's multiple range test was used to determine differences in means. Numbers in Tables I and II not sharing a superscript letter are significantly different.

Results. Body and relative tissue weights, hematocrit, and mortality of rats are presented in Table I. Body weight was increased by ovariectomy in the female rats from the fourth week of the study. Castration in the males did not affect growth rate. Heart weight was not affected by ovariectomy in females when compared to their intact controls. Similarly, castration did not affect heart weight of males. However, heart weight of males was significantly greater than that of females.

Liver weight was not affected by the treatment in either males or females when compared to that of their intact controls but was greater in males than in females. Adrenals were hypertrophied in females, but their weight was not affected by ovariectomy.

Hematocrit was significantly reduced in males compared with females, at 4 and 8 weeks of the study, and was further reduced in intact males.

Six of the intact and six of the castrated males were sacrificed at the end of the eighth week. Intact male rats began dying after being fed the diet for five weeks. By the ninth week 18 of the 37 rats had died. Castration delayed mortality by 2 weeks. The castrated rats began dying from the seventh week of the study. By

TABLE I. BODY AND RELATIVE TISSUE WEIGHTS, HEMATOCRIT, AND MORTALITY OF FEMALE AND MALE RATS FED A COPPER-DEFICIENT DIET FOR 4 AND 8 WEEKS[a]

	Females		Males	
	Intact	Ovariectomized	Intact	Castrated
4 weeks				
Body wt (g)	150 ± 3	170 ± 5	175 ± 5	177 ± 5
Hematocrit (%)	41 ± 1[b]	34 ± 2[c]	17 ± 1[d]	36 ± 2[c]
8 weeks				
Body wt (g)	171 ± 3	196 ± 10	191 ± 9	206 ± 11
Heart wt (g/100)	0.41 ± 0.007	0.50 ± 0.02	0.61 ± 0.05	0.63 ± 0.07
Liver wt (g/100)	3.4 ± 0.2	3.5 ± 0.2	3.9 ± 0.2	3.9 ± 0.1
Adrenals wt (g/100)	0.04 ± 0.002	0.04 ± 0.002	0.03 ± 0.003	0.03 ± 0.001
Hematocrit (%)	42 ± 1[b]	38 ± 2[b]	17 ± 2[c]	30 ± 2[d]
Mortality	0/12	0/12	18/37	4/12

	ANOVA[e] (*P* value)		
	Sex	Gonadectomy	Sex × gonadectomy
4 weeks			
Body wt	NS	0.04	NS
Hematocrit	0.0001	NS	0.0002
8 weeks			
Body wt	NS	0.04	NS
Heart wt	0.001	NS	NS
Liver wt	0.01	NS	NS
Adrenals wt	0.08	NS	NS
Hematocrit	0.001	0.03	0.0007

Note. NS, not significant.
[a] Each value represents the mean ± SEM of six rats per group.
[b,c,d] Indicates significantly different.
[e] A 2 × 2 analysis of variance.

the ninth week, 4 of the 12 castrated rats died. By the eleventh week of the study all remaining rats, castrated and intact had died. In contrast, none of the females, regardless of treatment, died (Table I).

Table II summarizes plasma levels of estrogens and testosterone at time of sacrifice. Higher levels of testosterone were found in males than in females. In the male rat, castration reduced the levels of testosterone by approximately 50% when compared to those of intact controls. The ovariectomy of females significantly reduced plasma levels of total estrogen by 48% when compared to those of their intact controls.

Hepatic and plasma copper concentrations, erythrocyte SOD, and plasma ceruloplasmin activities are presented in Table III. Hepatic copper was affected by the sex of the animal. Regardless of treatment, females had reduced hepatic copper concentration compared with males ($P < 0.03$). Ceruloplasmin activity was non-detectable in all copper-deficient rats. Plasma copper concentration and erythrocyte SOD activity were affected neither by the sex of the animal nor by the treatment.

Discussion. In agreement with our previous data (1), the present study shows that intact females are protected against the fructose-induced mortality of copper deficiency. In addition, ovariectomized females are also protected against the severity of copper deficiency since the ovariectomy of the female reduced total plasma estrogen by 48%, but this change had no effect on the symptoms of copper deficiency. Thus, regardless of whether females are ovariectomized or intact they are not susceptible to the severity of copper deficiency, suggesting that estrogens do not affect the symptoms associated with copper deficiency. Gonadectomized females and males had similar levels of estrogen and testosterone. However, castration of the male reduced testosterone levels by 50%, improved the copper-deficiency anemia, and delayed the onset of death by 2 weeks when compared with that of intact males. Thus, the reduced testosterone levels in the castrated rats appeared to ameliorate the severity of copper deficiency. However, this protection was only temporary, since even the castrated males died of the deficiency by the eleventh week of the study.

TABLE II. PLASMA LEVELS OF TESTOSTERONE AND TOTAL ESTROGENS OF MALE AND FEMALE RATS FED A COPPER-DEFICIENT DIET FOR 8 WEEKS[a]

	Testosterone (pmole/liter)	Estrogens (pmole/liter)
Females		
Intact	160 ± 10^{b}	212 ± 31^{c}
Ovariectomized	100 ± 30^{c}	103 ± 7^{b}
Males		
Intact	200 ± 20^{d}	$146 \pm 20^{b,c}$
Castrated	100 ± 10^{c}	126 ± 19^{b}
	ANOVA[e] (*P* value)	
Treatment		
Sex	0.006	NS
Gonadectomy	0.001	0.006
Sex × gonadectomy	0.009	NS

Note. NS, not significant.
[a] Each value represents the mean ± SEM for six rats.
[b,c,d] Indicates significantly different.
[e] A 2 × 2 analysis of variance.

Although gonadectomy reduced the levels of testosterone and estrogens in male and female rats, respectively, levels of testosterone and estrogens during the eighth week of the study show that all the rats were still in their prepubertal stage. Feeding a fructose-based diet from weaning has been reported to delay testicular development compared with that of rats fed starch (12).

It has been suggested that the high levels of sex hormones at birth or immediately after birth are of importance in "programming" the central nervous system and hypothalamic function (13–17). Sex-related differences in physiological and metabolic pathways have been predetermined and pre-regulated by the exposure of the hypothalamo–pituitary–gonadal axis to sex hormones during fetal and neonatal development (13–17). This phenomenon may explain the results of the present study, in which the sex of the rat determines the severity of copper deficiency.

In addition to differences in the levels of sex (13–17) and growth hormones (18) due to gender, a variety of metabolic pathways are also gender related. It is well established that there is a sexual dimorphism of absorption of iron (19), induction of fatty liver (20), dietary-induced cirrhosis (21), uric acid metabolism (22), α-tocopherol requirements (23), and glutathione peroxidase and reductase activities (24).

TABLE III. HEPATIC AND PLASMA COPPER AND CERULOPLASMIN ACTIVITY AND ERYTHROCYTE SOD OF MALE AND FEMALE RATS FED A COPPER-DEFICIENT DIET FOR 8 WEEKS[a]

	Females		Males	
	Intact	Ovariectomized	Intact	Castrated
Hepatic copper (μg/g wet wt)	0.87 ± 0.17	1.02 ± 0.19	1.68 ± 0.30	1.19 ± 0.17
Ceruloplasmin (U/liter)	ND	ND	ND	ND
Plasma copper (μmol/liter)	1.6 ± 0.5	2.8 ± 0.3	2.4 ± 0.4	2.7 ± 0.4
Erythrocyte SOD (U/ml)	14.3 ± 3.5	14.3 ± 8.8	8.9 ± 4.5	10.2 ± 1.0

	ANOVA[b] (*P* values)		
	Hepatic Cu	Plasma Cu	Erythrocyte SOD
Treatment			
Sex	0.03	NS	NS
Gonadectomy	NS	NS	NS
Sex × gonadectomy	NS	NS	NS

Note. NS, not significant; ND, nondetectable.
[a] Each value represents the mean ± SEM for six rats.
[b] A 2 × 2 analysis of variance.

Numerous studies have demonstrated a gender-linked difference in the basal activities of lipogenic enzymes, such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (25–27). The activities of these enzymes are more than twice as high in females as in males (28). In addition, a generalized disaccharide effect could not be demonstrated with female rats (29) as was previously reported with male rats (28).

Men and postmenopausal women manifest abnormally high levels of circulating triglycerides and fatty acids after being fed diets containing fructose (30). However, this response is absent in females of reproductive age (31). If fructose is metabolized differently in males than in females, it may be that copper requirements will also be gender dependent. The findings that male rats fed a copper-deficient diet containing starch are not anemic and that they do not die of the deficiency (5) may support the hypothesis that upon fructose feeding a certain metabolite of fructose could interact with copper deficiency, but only in males. However, this metabolite is absent when starch is fed.

Although it has been reported that estrogens increase ceruloplasmin activity in females (4) this could not be demonstrated in the present study. It is suggested either that in copper deficiency the incorporation of copper into ceruloplasmin is inhibited or that the levels of estrogens in the intact prepubertal female are too low to stimulate ceruloplasmin synthesis.

Hepatic copper concentration is used as an indicator of copper status. Females had lower levels of hepatic copper than males, indicating a reduced copper status. However, since the levels of hepatic copper of intact males were double the levels of intact females, but the males died of the deficiency, it is suggested that the mortality of copper-deficient males may not be solely due to the levels of hepatic copper. In addition, castrated males had lower levels of hepatic copper than their intact controls but their mortality was delayed. Furthermore, when copper-deficient males are fed starch, their hepatic copper concentrations are similar to those of copper-deficient rats fed fructose, but they survive (1). Thus, our previous data (1) and the results of the present study stress the point that unless fructose is fed to a copper-deficient male rat, copper deficiency per se is not sufficient to produce anemia, heart hypertrophy and pathology, and mortality.

1. Fields M, Lewis C, Scholfield D, Powel AS, Rose AJ, Reiser S, Smith JC. Female rats are protected against the fructose-induced mortality of copper deficiency. Proc Soc Exp Biol Med **183**:145–149, 1986.
2. Russanov E, Banskalieva V, Ljutakova S. Influence of sex hormones on the subcellular distribution of copper in sheep liver. Res Vet Sci **30**:233–235, 1981.

3. Saylor WW, Downer JV. Copper and zinc distribution in the liver and oviduct of estrogen and testosterone treated hens. Nutr Res **6**:181–190, 1986.
4. Carruthers ME, Hobbs CB, Warren RL. Raised serum copper and ceruloplasmin levels in subjects taking oral contraceptives. J Clin Pathol **19**:498–504, 1966.
5. Fields M, Holbrook J, Scholfield D, Rose A, Smith JC, Reiser S. Development of copper deficiency in rats fed fructose or starch: Weekly measurements of copper indices in blood. Proc Soc Exp Biol Med **181**: 120–124, 1986.
6. Schosinsky KH, Lehmann HP, Beeler MF. Measurements of ceruloplasmin and its oxidase activity in serum by use of *O*-dianisidine dihydrochloride. Clin Chem **20**:1556–1563, 1974.
7. Radioassay Systems Laboratories, Inc. ^{125}I Testosterone catalog number 1102 and ^{125}I Total Estrogens. In: Catalog No. 1022. Carson, CA, Radioassay Systems Laboratories, Inc., 1983.
8. Misra HP, Fridovich I. Superoxide dismutase: A photochemical augmentation assay. Arch Biochem Biophys **181**:308–312, 1977.
9. Hill AD, Patterson KY, Veillon C, Morris ER. Digestion of biological materials for mineral analysis using a combination of wet and dry ashing. Anal Chem **58**: 2340–2342, 1986.
10. Perkin–Elmer Corporation. Analytical Methods for Atomic Absorption Spectrophotometry. Publ. 303-0152. Norwalk, CT, Perkin–Elmer Inc., 1976.
11. SAS Institute, Inc. SAS User's Guide: Statistics, version 5 ed. Cary, NC, SAS Institute, Inc, 1985.
12. Kennedy BW, Working PK, Bhathena SJ, Lewis CG, Fields M. The effect of dietary carbohydrate and copper status on testicular development (abstract). Fed Proc **46**, 1986.
13. Arai Y, Gorski RA. Critical exposure time for androgenization of the rat hypothalamus determined by antiandrogen injection. Proc Soc Exp Biol Med **127**: 90–593, 1968.
14. Forest MG, Sizonenko PC, Cathiard AM, Bertrand J. Hypophyso-gonadal function in humans during the first year of life. I. Evidence for testicular activity in early infancy. J Clin Invest **53**:819–828, 1974.
15. Johnson TR. Development of the testes. In: Johnson TR, Moore WM, Jeffries JF, Eds. Children Are Different: Developmental Physiology. Columbus, OH, Ross Laboratory, pp113–119, 1978.
16. Faiman C, Winter JSD. Sex differences in gonadotrophin concentrations in infancy. Nature (London) **232**: 130–31, 1971.
17. Reiter EO, Root AW. Function of the pituitary gland. In: Johnson TR, Moore WM, Jeffries JF, Eds. Children Are Different: Developmental Physiology. Columbus, OH, Ross Laboratory, pp69–73, 1978.
18. Ho KY, Leong DA, Sinha YN, Johnson ML, Evans WS, Thorner MO. Sex related differences in GH secretion in rat using reverse hemolytic plaque assay. Amer J Physiol **250**E:650–654, 1986.
19. Guiden N. The influence of age and sex on the transduodenal transport of iron in the rat. In: Mills CF, Bremner I, Chesters JK, Eds. Trace Elements in Man and Animals—TEMA 5. Farnham Royal, Slough, United Kingdom, Commonwealth Agricultural Bureaux, pp521–523, 1985.
20. Sidransky H, Farber E. Sex differences in induction of periportal fatty liver by methionine deficiency in the rat. Proc Soc Exp Biol Med **98**:293–297, 1958.
21. Reuben M. Influence of age and sex on dietary-induced cirrhosis. Arch Environ Health **18**:792–797, 1969.
22. Anton FM, Puig JG, Ramos T, Gonzales P, Ordas J. Sex differences in uric acid metabolism in adults: Evidence for a lack of influence of estradiol-17B (E_2) on the renal handling of urate. Metabolism **35**:343–348, 1986.
23. Ward RJ. Sex differences in the tocopherol requirements of rats as shown by the haemolysis test. Brit J Nutr **17**:135–139, 1963.
24. Pinto RE, Bartley W. The nature of the sex-linked differences in glutathione peroxidase activity and aerobic oxidation of glutathione in male and female rat liver. Biochem J **115**:449–456, 1969.
25. Glock GE, McLean P. A preliminary investigation of the hormonal control of the hexose monosphosphate oxidative pathway. Biochem J **61**:390–397, 1955.
26. Hunter F, Hayes GW. Interaction of tissue glucose-6-phosphate dehydrogenase (G6PD) activity with age and sexual development in the rat. Endokrinologie **54**:85–96, 1969.
27. Taylor CB, Bailey E, Bartley W. Changes in hepatic lipogenesis during development of the rat. Biochem J **105**:717–722, 1967.
28. Lee VM, Szepesi B, Hansen RJ. Gender linked differences in dietary induction of hepatic glucose-6-phosphate dehydrogenase, 6 phophogluconate dehydrogenase and malic enzyme in the rat. J Nutr **116**: 1547–1554, 1986.
29. Lee VM, Szepesi B, Hansen RJ. Absence of a generalized disaccharide effect in adult female rats. J Nutr **116**:1555–1560, 1986.
30. MacDonald I. Influence of fructose and glucose on serum lipid levels in men and pre- and post-menopausal women. Amer J Clin Nutr **18**:369–372, 1966.
31. Reiser S, Bickard MC, Hallfrisch J, Michaelis OE IV, Prather ES. Blood lipids and their distribution in lipoproteins in hyperinsulinemic subjects fed three different levels of sucrose. J Nutr **111**:1045–1057, 1981.

Received March 17, 1987. P.S.E.B.M. 1987, Vol. 186.
Accepted June 29, 1987.