Blood Risk Factor Metabolites Associated with Heart Disease and Myocardial Fatty Acids in Copper-Deficient Male and Female Rats (42923)

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Abstract. **Intact and castrated males and intact and ovariectomized female rats were fed a copper-deficient diet in order to establish whether the protection provided in females against cardiovascular pathology and mortality is due to endogenous sex hormones, and different levels of blood lipids and/or myocardial fatty acids. Seventy-three male and female rats were assigned to a copper-deficient diet (0.6 μg of copper/g diet) containing 62% fructose for 8 weeks. Twelve of the male rats underwent castration and 12 of the females were ovariectomized. All animals exhibited high levels of plasma cholesterol, triglycerides, and uric acid, which were neither affected by the sex of the rat nor by the surgical treatment. The composition of fatty acids of the myocardium was similar in males and females. Except for those animals that were sacrificed by us, all other male rats died of heart pathology. In contrast, none of the female rats exhibited heart pathology and none died of the deficiency. It is suggested that heart pathology and mortality in copper deficiency are sex related and not due to high levels of plasma cholesterol, triglycerides, and uric acid or to differences in myocardial fatty acid composition.** [P.S.E.B.M. 1989, Vol 191]

Metabolic risk factors associated with heart disease include elevated fasting blood triglycerides (1, 2), cholesterol (3), and uric acid (4–6). The same blood risk factor metabolites associated with heart disease have been shown to be elevated by the feeding of fructose-containing sugars (7) and by copper deficiency (8–10). The copper-deficient animals that exhibited high levels of those metabolites also exhibited heart pathology which eventually led to their mortality (8–10). In hearts of rats fed a copper-deficient diet containing fructose or sucrose, there is a marked ventricular hypertrophy and mild to severe myocardial inflammation, degeneration, and fibrosis (11). Aneurysms of the left ventricle and pericarditis are also common (11).

The risk of heart disease is not randomly distributed throughout the population. It increases with age, and the disease affects males to a greater degree than females (12, 13). In contrast to men and postmenopausal women (14, 15), no increases in blood triglycerides and cholesterol were observed in young women after they had consumed fructose-containing diets (16, 17). Women have also been shown to have significantly lower serum levels of uric acid as compared with men (18).

Independent of changes in blood lipids, differences in fatty acid composition of cardiac tissue may be gender dependent (19). Alterations in double bonds of cardiac membranes may affect membrane fluidity, receptor binding, intrinsic enzymes, or ion transport properties (20, 21), which in turn may lead to cardiovascular lesions. Recent studies have demonstrated gender-linked effects in the basal activities of lipogenic enzymes in response to starvation and refeeding of rats (22, 23), a finding that may indicate differences in metabolic pathways.

We have recently reported that in contrast to males, female rats are protected against heart hypertrophy, pathology, and mortality when fed copper-deficient diets (24, 25). If blood lipids and fatty acids profile of

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heart tissue are influenced by endogenous sex hormones, the gonadectomy of the male and female animal can be either beneficial or lethal to the animal.

Intact and gonadectomized rats were used in this study in order to establish whether the protection provided in female rats against cardiovascular pathology and mortality is due to different levels of blood lipids and different composition of myocardial fatty acids.

Materials and Methods

Detailed descriptions of the materials and methods of this study were included in a preceding publication (25). Briefly, a total of 73 weanling male and female Sprague-Dawley rats weighing approximately 45–55 g each were purchased from Harlan-Sprague Dawley, Indianapolis, IN. Animals were divided into the following four groups: Group 1, female intact ($n = 12$); Group 2, female ovariectomized ($n = 12$); Group 3, male intact ($n = 37$); and Group 4, male castrated ($n = 12$). The 12 females were ovariectomized at Day 16 by the supplier, but the 12 males were castrated in our laboratory on arrival (Day 21). The absence of ovaries was verified at sacrifice 8 weeks later. All rats were fed from weaning a copper-deficient diet (0.6 μg of copper/g diet) containing fructose for 8 weeks. The diet contained the following (g/kg diet): carbohydrate as fructose, 627; egg white, 200; corn oil, 95; nonnutritive fiber (cellulose), 30; AIN-76 salt mix, 35 (26) (prepared in our laboratory and formulated to either omit or include cupric carbonate); and AIN-76A vitamin mix, 10 (27) supplemented with 2 mg of biotin and 2.7 mg of choline bitartrate. At the end of the eighth week, six rats from each group were killed by decapitation. The levels of total estrogens and testosterone were measured in plasma (28). Plasma cholesterol, triglycerides, and uric acid were analyzed using the enzymatic automated system of Centrifichem. Ceruloplasmin activity was measured in plasma (29). Hearts were removed, homogenized, the lipid extracted and separated into neutral and phospholipid fractions, and the fatty acids were separated by gas liquid chromatography as described previously (30).

Copper concentrations in liver were measured following their digestion by combining heat and dry ashing (31). The concentration of copper in plasma was also analyzed. To assure accuracy, the National Bureau of Standards Certified Reference Materials were digested and analyzed along with tissues by atomic absorption spectrophotometry (32). Data were analyzed by analysis of variance (ANOVA) using the SAS software system for data analysis (33). A $P < 0.05$ was considered as statistically significant.

Results

None of the female rats died during the study. In contrast, intact male rats began dying after the copper-deficient diet had been fed for 5 weeks. Except for the six intact and six castrated males that were sacrificed by us, 18 of the 37 intact rats died and 4 of the 12 castrated males died by the eighth week of the study. However, castration delayed mortality of male rats by 2 weeks. By the 11th weeks, all remaining male rats, intact and castrated, died. Body weight was not affected by the sex of the rat, but gonadectomy increased weight gain compared with intact controls (Table I). Regardless of castration, all hearts from male rats were enlarged and pale. Heart weight and heart weight to body weight ratios were increased in males when compared with females. The increased size was not due simply to cardiac dilation and general flabbiness of the ventricles, but the myocardium was hypertrophied, the walls thickened, and aneurysms were found at the ventricular

Table I. Heart Weight, Hematocrit, and Plasma Levels of Cholesterol, Triglycerides, and Uric Acid of Male and Female Rats Fed a Copper-Deficient Diet for 8 Weeks

	Females		Males	
	Intact	Ovariectomized	Intact	Castrated
Body weight (g)	171 ± 3[a]	196 ± 10	191 ± 9	206 ± 11
Heart weight (g/100 g)	0.41 ± 0.007	0.5 ± 0.02	0.61 ± 0.05	0.63 ± 0.07
Hematocrit (%)	42 ± 1	38 ± 2	17 ± 2	30 ± 2
Cholesterol (mg/dl)	130 ± 6	133 ± 8	150 ± 26	156 ± 16
Triglycerides (mg/dl)	130 ± 15	140 ± 11	182 ± 39	186 ± 19
Uric acid (mg/dl)	10 ± 2	7 ± 1	6 ± 1	9 ± 1
ANOVA	Sex	Gonadectomy	Sex × gonadectomy	
Body wt (g)	NS[b]	0.04	NS	
Heart weight (g/100 g)	0.001	NS	NS	
Hematocrit (%)	0.001	0.03	0.0007	
Cholesterol (mg/dl)	NS	NS	NS	
Triglycerides (mg/dl)	NS	NS	NS	
Uric acid (mg/dl)	NS	NS	NS	

[a] Results are expressed as mean ± SEM of six observation groups. A 2 × 2 analysis of variance.
[b] NS = not significant.

apex. In contrast, all hearts from female rats were normal in appearance and none were hypertrophied.

Ceruloplasmin activity was nondetectable in all animals regardless of sex or treatment. The mean ± SEM for plasma copper concentration of all animals was 2.3 ± 0.27 μmol/liter. The levels of hepatic copper concentrations for females intact and ovariectomized and male intact and castrated were: mean μg/g wet ± SEM of six observations per group 0.87 ± 0.17, 1.02 ± 0.19, 1.68 ± 0.30, and 1.19 ± 0.17, respectively. Hepatic copper concentrations were significantly affected by the sex of the animal ($P < 0.03$), females having lower concentrations than males. However, hepatic copper concentrations were neither affected by gonadectomy nor by the interaction between sex × gonadectomy.

The levels of total estrogens in plasma of ovariectomized females (103 ± 7 pmol/liter) were significantly reduced when compared with intact controls (212 ± 31). In the male rat, castration reduced plasma concentrations of testosterone (100 ± 10 pmol/liter) when compared with intact animals (200 ± 21).

Plasma concentrations of cholesterol, triglycerides, and uric acid were neither affected by the sex of the animal nor by gonadectomy. Only males were anemic. The anemia was ameliorated by castration (Table I).

Fatty acid composition of neutral lipids is presented in Table II. Higher levels of 15:0 and 16:1, but lower levels of 22:6 were found in males as compared with females. Regardless of treatment, higher levels of 14:0 and 18:0 were found in females as compared with males. Gonadectomy increased levels of 20:3. The fatty acids of 18:2, 20:0, and 24:0 chain length were affected by sex × gonadectomy.

Table III summarizes data of fatty acid composition of the phospholipid fraction. Except for higher levels of 16:1 and lower levels of 20:3 in females as compared with males, the composition of all other fatty acids were not affected by the sex of the rat. Gonadectomy increased levels of 16:1 and 18:1 but decreased 18:0. Only 15:0 was affected by sex × gonadectomy. The sum of all unsaturated fatty acids was increased by gonadectomy ($P < 0.002$). Intact females had the highest levels of unsaturation compared with males ($P < 0.04$).

Discussion

The results of this study have shown that regardless of gonadectomy and the sex of the animal, all rats exhibited similar concentrations of blood cholesterol, triglycerides, and uric acid. However, since the females of this study neither developed heart pathology nor died of the deficiency, it may be that the reason for the male mortality is not directly related to the increases in blood lipids. The possibility that it is the distribution of cholesterol in the lipoprotein rather than total cholesterol that determines and exacerbates heart pathology cannot be ruled out. Since the cholesterol moiety in plasma was not further separated into the lipoprotein components, it is difficult to argue against such an hypothesis. However, since unlike humans, the predominant lipoprotein fraction carrying cholesterol in rat plasma is HDL cholesterol rather than LDL cholesterol, the separation of plasma lipoproteins may not be helpful in this matter. In this respect, rat studies may not be expanded too far to include conditions in human beings.

Table II. Composition of Fatty Acids from Heart Neutral Lipid Fraction[a]

	Females[b]		Males[c]		ANOVA
	Ovariectomized	Intact	Castrated	Intact	
14:0	0.53 ± 0.05	0.82 ± 0.16	1.13 ± 0.14	1.47 ± 0.29	Sex
15:0	0.37 ± 0.04	0.39 ± 0.06	0.51 ± 0.08	0.54 ± 0.09	Sex
16:0	20.27 ± 0.21	20.67 ± 0.40	21.15 ± 0.87	19.95 ± 0.74	
16:1	1.98 ± 0.18	2.20 ± 0.07	2.61 ± 0.23	2.41 ± 0.21	Sex
18:0	14.17 ± 0.90	16.17 ± 1.02	16.00 ± 2.75	11.80 ± 0.69	Sex
18:1	26.49 ± 1.07	28.20 ± 0.89	28.05 ± 1.88	25.39 ± 0.94	
18:2	27.83 ± 1.30	24.39 ± 0.81	23.12 ± 4.44	29.99 ± 0.89	Sex × gonadectomy
18:3	0.07 ± 0.05	0.09 ± 0.09	0.22 ± 0.11	0.14 ± 0.05	
20:0	0.15 ± 0.05	0.24 ± 0.01	0.30 ± 0.07	0.20 ± 0.01	Sex × gonadectomy
20:3	0.33 ± 0.01	0.27 ± 0.02	0.32 ± 0.02	0.31 ± 0.01	Gonadectomy
20:4	5.68 ± 0.24	4.82 ± 0.56	4.51 ± 0.92	5.69 ± 0.55	
20:5	0.08 ± 0.02	0.11 ± 0.02	0.07 ± 0.03	0.11 ± 0.08	
22:5	0.49 ± 0.09	0.44 ± 0.05	0.89 ± 0.42	0.45 ± 0.10	
22:6	0.51 ± 0.05	0.45 ± 0.05	0.32 ± 0.07	0.38 ± 0.06	Sex
24:0	0.21 ± 0.21	0.00 ±	0.01 ± 0.01	0.83 ± 0.32	Sex × gonadectomy
24:1	0.84 ± 0.17	0.74 ± 0.12	0.80 ± 0.17	0.33 ± 0.21	
Saturated	64.3 ± 0.32	61.7 ± 0.28	60.9 ± 0.28	65.2 ± 0.21	Sex × gonadectomy
Nonsaturated	35.7 ± 0.24	38.3 ± 0.33	39.1 ± 0.56	34.8 ± 0.31	Sex × gonadectomy

[a] Percentage of composition of fatty acids from total neutral lipids from six rats/group.
[b] Values are mean ± SEM.
[c] A 2 × 2 analysis of variance. A probability of at least 0.05 was considered significant and is given for main treatment effects and interactions.

Table III. Composition of Fatty Acids from Heart Phospholipid Fraction[a]

	Females		Males		ANOVA
	Ovariectomized	Intact	Castrated	Intact	
12:0	0.03 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.05	
14:0	0.11 ± 0.07	0.47 ± 0.40	0.19 ± 0.07	0.12 ± 0.04	
15:0	0.06 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.04	Sex × gonadectomy
16:0	17.27 ± 0.63	17.79 ± 1.24	14.92 ± 1.02	16.51 ± 1.31	
16:1	0.86 ± 0.10	0.46 ± 0.09	0.57 ± 0.13	0.33 ± 0.07	Sex × gonadectomy
18:0	39.54 ± 0.75	48.21 ± 2.38	44.80 ± 2.25	47.23 ± 1.50	Gonadectomy
18:1	23.29 ± 3.59	12.00 ± 1.29	16.45 ± 2.99	12.21 ± 0.49	Gonadectomy
18:2	10.01 ± 1.99	10.73 ± 0.91	9.84 ± 2.19	14.51 ± 1.44	
18:3	0.20 ± 0.09	0.24 ± 0.12	0.24 ± 0.12	0.24 ± 0.21	
20:0	0.61 ± 0.09	0.78 ± 0.28	0.59 ± 0.11	0.62 ± 0.15	
20:3	0.23 ± 0.02	0.23 ± 0.09	0.47 ± 0.10	0.35 ± 0.09	Sex
20:4	6.02 ± 1.09	6.20 ± 1.58	7.22 ± 1.72	5.12 ± 0.73	
20:5	0.20 ± 0.13	0.21 ± 0.16	0.95 ± 0.50	0.25 ± 0.13	
22:5	0.28 ± 0.16	0.73 ± 0.41	0.64 ± 0.55	0.70 ± 0.30	
22:6	0.50 ± 0.05	0.63 ± 0.17	1.04 ± 0.31	0.58 ± 0.21	
24:0	0.40 ± 0.15	0.83 ± 0.47	1.73 ± 0.71	1.00 ± 0.76	
24:1	0.38 ± 0.14	0.49 ± 0.18	0.37 ± 0.17	0.69 ± 0.23	
Total					
Saturated	58.0 ± 0.9	68.1 ± 2.4	62.2 ± 1.0	65.0 ± 1.2	Gonadectomy
Unsaturated	42.0 ± 0.9	31.9 ± 2.4	37.8 ± 1.0	35.0 ± 1.1	Gonadectomy

[a] See footnote *a* to Table II.

The literature regarding gender differences in carbohydrate metabolism is limited. However, it has been shown that fructose-containing sugars had been metabolized differently in male than in female rats under starvation refeeding conditions (22, 23). In addition, it has been recently shown that fructose metabolism is gender dependent (34). Sorbitol and glyceraldehyde accumulated in livers and kidneys of male rats consuming fructose when compared with female rats (34). Sorbitol is a strong chelator of copper (35). The sorbitol copper complex is very stable and does not dissociate easily (35). The increased intracellular levels of sorbitol may chelate the limited levels of copper in the copper-deficient rat consuming fructose, making the chelated copper unavailable for utilization and exacerbating the copper deficiency. Glyceraldehyde, an intermediate in fructose metabolism and a precursor in lipogenesis, is toxic to tissues since it undergoes auto-oxidation and it generates free radicals (36). Female rats consuming fructose did not have elevated tissue sorbitol and glyceraldehyde concentrations, and thus should be protected from the considerable symptoms associated with the deficiency in fructose fed males.

Regardless of sex and treatment, copper concentration in plasma was similar in all animals. However, when hepatic copper concentration was used to assess copper status, females had lower copper status than males and yet they were protected against heart pathology. Only male rats were anemic. The anemia could contribute to heart hypertrophy (37). This hypothesis is further supported by the finding that anemia was absent in female rats, but only females were protected against heart hypertrophy and pathology. Iron deficiency anemia causes cardiac hypertrophy and copper deficiency also causes anemia (but only when the diet contains fructose) (37–41). However, the cardiac enlargement of copper deficiency occurs prior to the onset of anemia and is greater than that associated with iron deficiency (39, 41–43). In experiments in which iron and copper deficiency were imposed simultaneously, the anemia was not more severe than with iron deficiency alone and the cardiomegaly was no greater than with the copper deficiency alone (42). Thus, although anemia may not be a major factor in the cardiomegaly of copper deficiency, the additional burden of the anemia with copper deficiency may be an important factor in the development of myocardial pathology.

The role that copper plays in lipid metabolism and in the desaturation of fatty acids has been controversial. The supplementation of copper increased (44, 45), but copper deficiency decreased the desaturation of fatty acids (46, 47). In contrast, others found opposite (48–50) or variable effects (51). The reasons for the inconsistency are due to variations in the strain of experimental animals (44–53), reduced food intake (52), pair feeding (52), and the mode of induction of dietary copper deprivation (51). In this study, only one diet containing one level of copper has been used. This study did not include a copper-adequate diet. In this way any variable that copper status could exert on desaturation or saturation of fatty acids had been eliminated. As a result, differences in fatty acid composition can be affected only by the sex of the animal or by gonadectomy.

Regardless of copper, changes in polyunsaturated fatty acids of the myocardial phospholipids could have

consequences for membrane properties and functions. Effects on contractile performance, membrane fluidity, and enzymatic properties may in turn result in alterations of contractile performance (20, 21). The composition of myocardial fatty acids of the neutral and phospholipid fractions in male and female rats were similar; however, the sum of the unsaturated fatty acids of the phospholipid moiety was the greatest in ovariectomized females. Since superoxide dismutase activity is further reduced by copper deficiency and fructose feeding (54), a greater degree of peroxidation of the unsaturated fatty acids is expected to occur. Ovariectomized females should be more susceptible to membrane damage than all other animals. However, pathology of the heart was confined only to males.

Recently we have shown that plasma atrial natriuretic peptides which play a role in cardiac function and which levels are regulated by the anterior pituitary gland and are elevated with congestive heart disease (55, 56) were 4-fold higher in male rats fed the copper-deficient fructose diet than in females (57). Ovariectomy or castration did not have any effect on plasma atrial natriuretic peptide levels (57). The increased atrial natriuretic peptide levels in the copper-deficient male rat fed fructose which correlated well with myocardial damage and mortality may be gender related.

It is well established that the risk of ischemic heart disease in the human population affects males to a greater degree than females (1, 58) and, in our studies, only male rats were susceptible to copper deficiency. However, although the abnormalities in the rat hearts bear only some resemblance to human cardiac pathologic entities, such as the idiopathic cardiomyopathies (59, 60) and alcohol cardiomyopathy (60), there also are important differences, in particular a minimal inflammatory component in the human myopathies. Also, the risk of heart disease in the human population is most commonly associated with males and females of reproductive age. In contrast, the rats used in these studies have not yet reached sexual maturity. In this regard, there is a need for experiments with animals involving interactions between dietary components and other risk factors such as age and sex.

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