EFFECT OF A DIETARY COPPER DEFICIENCY ON PLASMA FIBRINOLYTIC ACTIVITY IN MALE AND FEMALE MICE

Sean M. Lynch, Ph.D.¹ and Leslie M. Klevay, M.D.²

United States Department of Agriculture
Agricultural Research Service
Grand Forks Human Nutrition Research Center
Grand Forks, ND 58202

ABSTRACT

To investigate the effects of a dietary Cu deficiency on plasma fibrinolytic activity, groups (n=16) of adult male and female Swiss-Webster mice were fed Cu-supplemented (8.4 mg Cu/kg) or Cu-deficient (0.3 mg Cu/kg) diets ad libitum with deionized water for 43-49 days. Animals were exsanguinated under pentobarbital anaesthesia; platelet-poor plasma was prepared and assayed for euglobulin clot lysis time (ECLT). Plasma protein C (PC) and antithrombin III (AT-III) activities also were determined. No statistically significant effect of Cu deficiency on either PC or AT-III activity was observed but a highly significant (P<0.001) ECLT prolongation in both male and female deficient mice clearly demonstrated that the physiological clot-lysing mechanism must be impaired in these animals. These results may help to explain the thrombotic sequelae of a dietary Cu deficiency in mice.

KEY WORDS: Copper, Fibrinolysis, Protein C, Antithrombin III, Mice

INTRODUCTION

Thrombosis has been identified (1-5) as an important factor in the etiology of ischemic heart disease (IHD) and it has been shown that female mice fed a copper (Cu)-deficient diet exhibit extensive cardiovascular damage including massive occlusive atrial thromboses. The diet used in these experiments was introduced 30 years ago by Ball et al. (6) as an improvement of the thrombogenic diets then in use. Examination of the diet and the cardiovascular lesions it

¹Current address: Department of Nutrition, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115.

²Address correspondence and reprint requests to: Leslie M. Klevay, M.D., USDA, ARS, GFHNRC, University of North Dakota, PO Box 9034, Grand Forks, ND 58202-9034.
produced led to the suspicion that Cu deficiency was responsible for most of the pathology. Experimentation (7) confirmed this suspicion; the control diet was improved in comparison to earlier work (6) by supplementation with copper, iodine, manganese and zinc via the drinking water. These observations tend to support the hypothesis that a dietary Cu deficiency may be an important factor in the etiology of IHD (8-10).

Recent studies have shown (11,12) that Cu-deficient mice have significantly lower plasma coagulation factor V and VIII activities than those fed a Cu-supplemented diet. It is not clear how such a change in coagulation factor activities may contribute to the observed thrombotic lesions but these results suggest that the hemostatic defect responsible is not in the coagulation system. A similar paradox has been observed in a number of humans (13) in whom uncontrolled thrombosis may occur despite an impairment of the coagulation system. However, a number of studies have reported decreased fibrinolytic activity in survivors of acute myocardial infarction (14-20) and it has been demonstrated that patients with IHD exhibit defects of the fibrinolytic system (21-24). Thus, regulation of the fibrinolysis, rather than coagulation, may be a more important factor in the pathology of IHD. In the experiment reported here, measurement of euglobulin clot lysis time (ECLT) was performed on plasma from Cu-deficient and Cu-supplemented mice fed a thrombogenic diet to determine the influence of Cu status on fibrinolytic activity. Furthermore, since plasma coagulation factor V and VIII activities decrease in Cu-deficient mice (11,12), the activities of two important plasma coagulation inhibitors (protein C [PC] and antithrombin III [AT-III]; 25) also were measured.

METHODS

Male and female mice of the Taconic Swiss-Webster strain (Taconic Farms, Germantown, NY)3 were assigned to groups of 16 at six weeks of age. The diet (7,12) has been described in great detail. In brief, it is based (g/kg) on sucrose (575), lard (280), casein (80) and all known essential vitamins and minerals including cystine. In the present experiment, each kilogram of copper-supplemented diet contained CuSO4·5H2O, 39.28 (mg/kg); KI, 0.66 (mg/kg); MnSO4, 30.76 (mg/kg); and Zn(C2H3O2)2·2H2O, 167.82 (mg/kg) added via the salt mix. Copper sulphate was omitted from the copper-deficient diet and sucrose was substituted; the other minerals were included. The mice were fed the diet ad libitum with deionized water for 43-49 days. At the end of the experiment, mice were anesthetized with sodium pentobarbital and exsanguinated from the inferior vena cava. Blood (0.5 ml) was collected in microcentrifuge tubes containing 3.8% (w/v) trisodium citrate (0.026 ml) as anticoagulant (26). Platelet-poor plasma (PPP) was prepared by centrifugation of whole blood at 3000 g for 20 minutes in a refrigerated (4°C) centrifuge. An aliquot of PPP was removed for preparation of the euglobulin fraction and the remainder was stored at −70°C. Erythrocyte pellets were stored at −70°C.

3Mention of a trademark or proprietary product does not constitute a guarantee of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.
The euglobulin fraction was prepared from PPP as follows. Freshly isolated PPP (0.05 ml) was pipetted into labelled microcentrifuge tubes. Ice-cold deionized water (0.6 ml) and acetic acid (1 % (v/v); 0.01 ml) were added and the solution mixed. The tubes were then placed in the refrigerator (4°C) for 10 mins. After this incubation, the tubes were centrifuged (1600 g x 3 mins; 4°C) and the supernatant discarded. The open tubes were inverted on absorbent paper and left to drain any residual liquid. In addition, the interior of the tubes were carefully swabbed with a cotton-tipped applicator to ensure complete removal of the supernatant. Phosphate buffered saline, pH 7.2, (0.035 ml) was added to each tube and the contents mixed carefully to ensure complete dissolution of the euglobulin precipitate. After preparation of the euglobulin fraction, thrombin (5 NIH Units/ml; 0.025 ml) was added to each tube and the tubes incubated in a waterbath at 37°C. Clot formation occurred approximately 30 secs after thrombin addition and tubes were examined at 10 min intervals for clot lysis. Any samples which had not lysed after 120 mins were arbitrarily assigned a lysis time of 120 mins. All euglobulin clot lysis time (ECLT) determinations were performed in duplicate.

Plasma PC activity was assayed by a modification of the method of Walker et al. (27). PPP which had been stored at −70°C was thawed by 5-10 mins incubation at 37°C and an aliquot (0.012 ml) was transferred to a small plastic tube. Protein C activator (Calbiochem Catalog No. 539217; PROTAC - reconstituted with deionized water to give an activity of 0.75 Units/ml) was added (0.02 ml) and the mixture incubated at 37°C for 4 mins. Protein C substrate (Calbiochem Catalog No. 200284; BOC-Z-Leu-Ser-Thr-Arg-PNA hydrochloride-reconstituted with deionized water to give a concentration of 10.4 mg/ml) was added (0.05 ml) and incubation continued at 37°C for 5 mins. After addition of deionized water (2.0 ml) to the tube, the contents were mixed, centrifuged (2000 g x 5 mins) and the absorbance of the supernatant at 405 nm was measured.

AT-III activity was determined in PPP which had been stored at −70°C using a synthetic substrate assay (Dade Proteolytic Enzyme Detection System; Catalog No. B4232-21).

Calibration curves were prepared for PC and AT-III assays using pooled plasma obtained from mice fed Rodent Lab Chow 5001 (Purina Mills Inc., St. Louis, MO). The PC and AT-III activities observed in this pooled plasma were arbitrarily assigned a value of 100% and activities in the plasmas of experimental mice were measured relative to this standard.

Plasma ceruloplasmin (Cp) activity was measured by a standard method (28) in samples which had been stored at −70°C. Copper and iron in diet and liver were determined by flame atomic absorption spectrophotometry following H₂SO₄/HNO₃/H₂O₂ digestion (29) using National Institute of Standards and Technology citrus leaves (1572) and bovine liver (1577a) as standards.

Data were analyzed by two-way analysis of variance (ANOVA; 30). Scheffé contrast analyses were performed on data from any ANOVA with a significant (P<0.05) Cu x sex interaction.
RESULTS

Dietary analysis revealed 8.4 or 0.3 mg Cu/kg for the supplemented or deficient diet, respectively. One female mouse fed the Cu-deficient diet died on day 37 of the experiment and was found to have an enlarged heart (1.09 % body weight) with an external adherent blood clot.

Several indices of Cu status are reported in Table 1. Cu deficiency significantly (P<0.001) lowered hematocrits in both male and female Cu-deficient mice. A significant (P<0.001) Cu x sex interaction was found for heart size and a significant (P<0.05) exacerbation of the cardiac enlargement associated with Cu deficiency (P<0.001) was observed in Cu-deficient female mice. Significant independent effects of Cu and sex on Cp activity were recorded. Cu-deficient mice had lower Cp than Cu-supplemented mice (P<0.001) and female mice had lower Cp activity than males (P<0.01). Liver Cu was significantly (P<0.05) decreased by deficiency in female, but not male, mice. Liver Fe was significantly (P<0.001) higher in female, compared with male, and Cu-deficient, compared with Cu-adequate, mice. A significant (P<0.05) Cu x sex interaction was found with deficiency producing the largest increase in liver Fe in males and with females having the highest values.

TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>M-S</th>
<th>F-S</th>
<th>M-D</th>
<th>F-D</th>
<th>Cu*</th>
<th>Sex*</th>
<th>Cu x Sex*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (% PCV)</td>
<td>32.4</td>
<td>39.1</td>
<td>7.6</td>
<td>5.4</td>
<td>0.001</td>
<td>NS†</td>
<td>0.05</td>
</tr>
<tr>
<td>Heart size (g/100 g)*</td>
<td>0.47</td>
<td>0.45</td>
<td>0.77</td>
<td>1.00</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Cp (Units/l)</td>
<td>28.8</td>
<td>15.4</td>
<td>9.3</td>
<td>3.7</td>
<td>0.001</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Liver Cu (µg/g)**</td>
<td>4.6</td>
<td>11.9</td>
<td>5.7</td>
<td>6.4</td>
<td>0.001</td>
<td>0.001</td>
<td>0.05</td>
</tr>
<tr>
<td>Liver Fe (mg/g)**</td>
<td>0.37</td>
<td>1.69</td>
<td>2.03</td>
<td>2.83</td>
<td>0.001</td>
<td>0.001</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Results given as mean (standard error), 16 animals per group.
†P< values from 2-way ANOVA. Results of Scheffé contrast analyses given as superscripts (x,y,z) - means in the same row with different superscripts are significantly (P<0.05) different. NS, Not significant.
‡Reported as g/100 g body weight.
**Liver metals reported on dry weight basis.
Data from ECLT, PC and AT-III analyses are reported in Table 2. No statistically significant effects of either Cu status or sex were observed for PC and AT-III, but a highly significant (P<0.001) prolongation of ECLT was observed in both male and female Cu-deficient, compared with Cu-adequate, mice.

**TABLE 2**

Euglobulin Clot Lysis Time (ECLT) and Plasma Protein C (PC) and Antithrombin III (AT-III) Activities in Male (M) and Female (F) Mice Fed Copper-Supplemented (S) or Copper-Deficient (D) Diets

<table>
<thead>
<tr>
<th></th>
<th>M-S</th>
<th>F-S</th>
<th>M-D</th>
<th>F-D</th>
<th>Cu*</th>
<th>Sex*</th>
<th>Cu x Sex*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECLT (min)</td>
<td>41.5</td>
<td>45.0</td>
<td>117.3</td>
<td>90.0</td>
<td>0.001</td>
<td>NS†</td>
<td>0.05</td>
</tr>
<tr>
<td>PC (%) PNP</td>
<td>176</td>
<td>174</td>
<td>133</td>
<td>82</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AT-III (%) PNP</td>
<td>95</td>
<td>86</td>
<td>101</td>
<td>106</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Results given as mean (standard error), 16 animals per group.

†P< values from 2-way ANOVA. Results of Scheffé contrast analyses given as superscripts (x,y) - means in the same row with different superscripts are significantly (P<0.05) different. †NS, Not significant.

‡Reported as a percentage of the activity in a pooled normal mouse plasma (PNP) sample which was arbitrarily assigned a value of 100%.

**DISCUSSION**

Copper deficiency was confirmed in mice by observation of decreased hematocrit and plasma Cp activity, increased liver Fe and cardiac enlargement (Table 1). Although plasma Cp activity was decreased and liver Fe increased in Cu-deficient male mice, there was no statistically significant effect of dietary Cu on liver Cu in male mice. In contrast liver Cu was significantly decreased in Cu-deficient, compared with Cu-supplemented, female mice. These results were somewhat similar to an earlier experiment (12) in which the largest change in liver copper was the decrease experienced by the deficient females. This change may contribute to the mechanism by which female mice are more susceptible to the lethal consequences of the Cu-deficient diet. That female mice experienced a more extreme form of Cu deficiency tends to be confirmed by the extreme cardiac enlargement, a recognized feature of Cu deficiency (31-33), observed in the Cu-deficient female mice.

Significant prolongation of ECLT, a measure of plasma total fibrinolytic activity (34,35), was found in both male and female Cu-deficient mice. This indicates that there is a severe
impairment of fibrinolysis in Cu-deficient mice. The main in vivo function of the fibrinolytic pathway is the generation of plasmin which facilitates thrombus dissolution (36,37). The impaired fibrinolytic system in the Cu-deficient mice may be the factor which results in the observed thrombotic lesions (6,7). In vivo plasmin activity is regulated by a number of interactive activator and inhibitor molecules (36,37). Recently, it has been suggested (38) that the plasma plasminogen activator inhibitor 1 (PAI-1) level is the most important factor in the determination of ECLT. Thus, it is possible that dietary Cu may mediate PAI-1 levels in plasma. However, further experiments will be necessary to investigate this hypothesis.

Although there were no statistically significant effects of either Cu status or sex on plasma PC and AT-III activities, a trend towards lower PC activity (P=0.08) was noted in both male and female Cu-deficient mice. PC is an important physiological coagulation inhibitor which limits coagulation by proteolytic degradation of coagulation factors V and VIII (25,39). Since it has been previously demonstrated that plasma coagulation factor V and VIII activities are significantly lower in Cu-deficient, compared with Cu-supplemented, mice (11,12) it may be permissible to speculate that such a decrease in PC activity may represent a homeostatic mechanism which enables the Cu-deficient mouse to maintain a functional coagulation system while experiencing the nutritional insult of Cu deficiency.

The results presented here may have implications for the pathophysiology of ischemic heart disease. The more extreme copper deficiency in female mice seems consonant with the greater likelihood of women than men with acute myocardial infarction found by Spikerman et al. (40) to die of thromboembolism. Patients with IHD have decreased fibrinolytic activity as measured by methods similar to the ECLT used here (14-17).

Although a daily Cu intake of 1.5-3.0 mg/day has been estimated as safe and adequate for adults (41), many diets may fail to provide this amount (8,9). Calculations based on 10 dietary surveys reveal that 35% of daily diets contain less than 1.0 mg of Cu (10). Amounts below 1.0 mg/day have been shown to be insufficient for more than 31 men and women in Cu depletion experiments (42-45). Furthermore, it has been proposed (8-10) that IHD etiology may be related to a dietary Cu deficiency. Therefore, the results of these dietary surveys and this investigation may be germane to the pathogenesis of IHD.

To summarize, this study demonstrated that dietary Cu is an important regulator of plasma total fibrinolytic activity which may represent the mechanism responsible for the thrombotic lesions observed in Cu-deficient mice (6).

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Denice Schafer & staff with diet preparation and animal care, Terry Wolf & Randy Perila for technical assistance, Sheila Bichler for statistical analyses, Marie Swenson for manuscript preparation.
REFERENCES


Accepted for publication May 18, 1993.