The Effects of Dietary Copper Deficiency and Psychological Stress on Blood Pressure in Rats

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KLEVAY, L. M. AND E. S. HALAS. The effects of dietary copper deficiency and psychological stress on blood pressure in rats. PHYSIOL BEHAV 49(2) 309–314, 1991.—In each of two experiments, adult, male Sprague-Dawley rats were deprived of copper and were subjected to the chronic stress of close confinement. A 2 × 2 factorial design was used because both copper deficiency and stress have been implicated in the regulation of blood pressure and are implicated in a major consequence of human hypertension— ischemic heart disease. Copper deficiency was verified by a decrease in copper in several organs. Both copper deficiency and stress increased blood pressure; results were independent. Sodium in heart was increased by deficiency in both experiments, but was increased in brain in only the second experiment. The combination of stress and deficiency produced an increase in mortality in one of two experiments. A decrease in cholesterol in plasma due to stress is consistent with earlier data from rats but is in contrast to data from humans. Both stress and copper deficiency produce potentially adverse changes in cardiovascular physiology and the chemistry of brain, heart and other organs. These results may be germane to humans because stress is frequent and some diets are low in copper.

<table>
<thead>
<tr>
<th>Cholesterol</th>
<th>Copper deficiency</th>
<th>Hypertension</th>
<th>Ischemic heart disease</th>
<th>Sodium</th>
<th>Stress</th>
</tr>
</thead>
</table>

A chronic increase in blood pressure is one of the prime predictors of risk of ischemic heart disease, the leading cause of death in the U.S. (4,19). There are numerous hormonal, metabolic, dietary, and neural factors that contribute to the regulation of blood pressure. The factors that stand out in the production of hypertensive illness in humans are more limited, however. Whelton and Patterson mention increased ingestion of salt, excessive caloric intake and emotional stress (52). Kaplan increases this triad by including a group of cooperating hormones and uses the word stress without an accompanying adjective (21).

Diets in the United States seem to be low in copper. The National Research Council of the National Academy of Sciences (3) estimated the safe and adequate daily intake of copper to be 2–3 mg. A recent estimate of daily copper intakes based on ten surveys in which dietary copper was measured by chemical analysis revealed that only 14% of daily intakes exceed 2 mg and 35% are below 1 mg (31).

The most important consequence of these diets is likely to be ischemic heart disease. First mentioned more than a decade ago (23,24), the association between copper metabolism and this illness has grown (26, 28, 31). Nearly 50 anatomical, chemical and physiological characteristics of people with ischemic heart disease can be attributed to insufficient copper. The possible contribution of copper deficiency to human hypertension has received recent emphasis (26,31). Adult rats deficient in copper have increased blood pressure (27,39); women fed diets low in copper respond to the stress of sustained hand grip exercise with an extra increase in blood pressure that is prevented by copper supplementation (37).

Although stress has long been implicated in hypertensive illness, its mechanism is unknown (10,15). Perhaps hypertension occurs only in combination with other factors such as salt intake, etc. (15, 17, 18) or in association with genetic predisposition (5, 15, 17, 18, 36). Page (44) has proposed the mosaic theory in which no single factor is uniquely responsible for hypertension; in contrast, combined effects of several factors produce illness.

This complexity involving stress has led to a search for a suitable animal research model. The use of aversive reinforcement in classical (8, 48, 49) and operand (9, 11, 12, 14, 16) conditioning paradigms did not consistently produce hypertension in

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2Requests for reprints should be addressed to Leslie M. Klevay, M.D., S.D. in Hyg., USDA, ARS, Grand Forks Human Nutrition Research Center, P.O. Box 7166, University Station, Grand Forks, ND 58202.
TABLE 1
COMPOSITION OF THE DIET (g)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>623.5</td>
</tr>
<tr>
<td>Egg white</td>
<td>200.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>90.0</td>
</tr>
<tr>
<td>Jones Foster salt mix*</td>
<td>40.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>30.0</td>
</tr>
<tr>
<td>Vitamin ADE mix†</td>
<td>10.0</td>
</tr>
<tr>
<td>Water soluble vitamin mix‡</td>
<td>5.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The details of the dietary formulation are published (31, 33, 49). This diet contains all nutrients known to be essential for rats. *Specially formulated without copper and zinc; these nutrients are supplied as drinking solutions (31, 42). †Alpha tocopherol, calciferol and vitamin A palmitate in corn oil. ‡Biotin, cyanocobalamin, folic acid, menadione, niacinamide, calcium pantothenate, pyridoxine, riboflavin and thiamin in sucrose.

normotensive animals. An avoidance-avoidance conflict procedure, a very potent stressor, raised blood pressure in normotensive rats only slightly (5); the same experimental procedures yielded much higher blood pressures (35) when rats with a borderline genetic susceptibility to hypertension were used. The use of rat strains with inherited hypertension has been useful (10, 42).

Our success (27) in raising blood pressure of rats with diets low in copper, the complexity of blood pressure regulation, and the prominence of stress in hypertension etiology prompted us to study rats subjected to stress and fed diets low in copper in all possible combinations: stress vs. no stress and supplemented vs. insufficient copper. We deliberately chose to use rats not known to be susceptible to hypertension to provide the most vigorous test of the hypothesis that stress can produce effects on blood pressure in addition to those of copper deficiency.

EXPERIMENT 1

METHOD

Animals and Diets

Sixty male weanling Sprague-Dawley rats (Harlan Sprague-Dawley, WI) were matched into four equal groups by mean (58 g) body weight. They were fed a purified diet (Table 1) based on 62% sucrose, 20% egg white and 10% corn oil (23, 25, 41). Although the diet contains all the nutrients known to be essential for rats, it is inadequate in copper and zinc and must be supplemented. Supplementation was provided as a drinking solution (23, 24) containing 10 μg zinc and 2 μg copper per ml (as acetate and sulfate, respectively). Rats were housed in individual cages of stainless steel under conditions similar to those described elsewhere (33).

Apparatus

Plexiglas restraint cages were used. These were made from a cylinder 20 cm long and 9 cm in diameter with a 3 mm wall. The cylinder was cut and a plane floor attached parallel to the axis so that the height of the cage was 6.7 cm. Slots were cut in the curved top of the cage at 12, 15, and 19 cm which allowed a guillotine door to be used to restrict the rat and prevent it from turning around. The restraint cages also were used when blood pressure was measured.

Chemical Analysis

Cholesterol in plasma was measured by fluorescence (6); copper, potassium, sodium and zinc in organs were measured by inductively coupled plasma emission spectrometry (ICP-6500, Perkin-Elmer Corp. Norwalk, CT) after digestion with nitric acid-hydrogen peroxide (40).

Procedures

When the rats reached a mean weight of 280 g, blood pressures (BP) were measured without anesthesia by the tail cuff method. The method has been described in detail (27, 31). Data were recorded with an Electro-Sphygmomanometer and pneumatic pulse transducer (Narco Bio-Systems, Houston, TX). Although no significant differences in blood pressure were found at this time, a few rats were exchanged between groups to make group blood pressures more similar. Then copper was omitted from the drinking solutions of the two groups with the lower blood pressures. Twenty-four days after copper deprivation was begun, stress in the form of physical restraint (45) was applied to half of the groups. A 2×2 factorial design was used. Group 1 was copper supplemented (CuS) and was not restrained (NR). Group 2 was copper supplemented and was restrained (R). Group 3 was copper deficient (CuD) and was not restrained while Group 4 was copper deficient and was restrained. For 23 consecutive days, the rats in Groups 2 and 4 were removed from their home cages starting at 9:00 a.m. and were placed prone in the restraint cages for 45 minutes in an adjoining room at 22°C. Then they were returned to their home cages. The blood pressures for all rats were measured weekly several hours after restraint. At the end of the experiment, all surviving rats were killed and organs were removed after intraperitoneal injection of sodium pentobarbital. Blood was obtained by cardiac puncture and saved for chemical analyses. Rats that died prematurely were examined for gross pathology. Organ and plasma data were analyzed using 2×2 analyses of variance (ANOVA) to test for effects of diet and restraint. Blood pressure data from surviving rats were analyzed using a repeated measures ANOVA which tested for effects of diet and restraint across the 3 and 4 weeks of Experiments 1 and 2, respectively. F-values and their associated degrees of freedom are

TABLE 2
BLOOD PRESSURE (mmHg)

<table>
<thead>
<tr>
<th>Week of Restraint</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Group 1: CuS, NR</td>
<td>121 ± 2.7</td>
<td>123 ± 3.6</td>
<td>118 ± 3.0</td>
<td>114 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>(15)</td>
<td>(15)</td>
<td>(15)</td>
<td>(15)</td>
</tr>
<tr>
<td>Group 2: CuS, R</td>
<td>122 ± 2.1</td>
<td>125 ± 1.7</td>
<td>126 ± 1.8</td>
<td>127 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>(15)</td>
<td>(15)</td>
<td>(15)</td>
<td>(14)</td>
</tr>
<tr>
<td>Group 3: CuD, NR</td>
<td>119 ± 2.9</td>
<td>122 ± 2.5</td>
<td>118 ± 2.0</td>
<td>119 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>(15)</td>
<td>(15)</td>
<td>(15)</td>
<td>(15)</td>
</tr>
<tr>
<td>Group 4: CuD, R</td>
<td>122 ± 2.5</td>
<td>123 ± 2.1</td>
<td>128 ± 2.1</td>
<td>131 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>(15)</td>
<td>(13)</td>
<td>(10)</td>
<td>(7)*</td>
</tr>
</tbody>
</table>

Mean ± S.E.; CuS = copper supplemented, CuD = copper deficient, NR = nonrestrained, R = restrained; (n) = number of surviving rats for that week. *Two rats died shortly after BP were recorded.
TABLE 3
COPPER IN ORGANS, µg/g, DRY WEIGHT

<table>
<thead>
<tr>
<th></th>
<th>Adrenal</th>
<th>Brain</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: CuS, NR</td>
<td>1.75 ± 0.29</td>
<td>9.0 ± 0.32</td>
<td>12.1 ± 1.12</td>
<td>11.4 ± 0.58</td>
<td>12.8 ± 0.88</td>
</tr>
<tr>
<td>Group 2: CuS, R</td>
<td>1.71 ± 0.09</td>
<td>8.7 ± 0.23</td>
<td>12.5 ± 0.87</td>
<td>12.0 ± 0.90</td>
<td>10.8 ± 1.15</td>
</tr>
<tr>
<td>Group 3: CuD, NR</td>
<td>0.75 ± 0.07</td>
<td>8.2 ± 0.23</td>
<td>8.3 ± 0.89</td>
<td>8.0 ± 0.32</td>
<td>3.4 ± 0.71</td>
</tr>
<tr>
<td>Group 4: CuD, R</td>
<td>0.71 ± 0.12</td>
<td>8.1 ± 0.23</td>
<td>9.1 ± 0.71</td>
<td>7.9 ± 0.25</td>
<td>6.5 ± 2.33</td>
</tr>
</tbody>
</table>

Mean ± S.E.; CuS = copper supplemented, CuD = copper deficient, NR = nonrestrained, R = restrained.

reported in the text for significant main effects and interactions (47).

RESULTS AND DISCUSSION

Analysis of variance of blood pressure was computed across time for rats surviving to week three. Data are shown in Table 2. The only significant main effect was that of restraint, F(1,47) = 15.0, p = 0.0003. Because the largest differences would be expected in the third week, an ANOVA was computed for that week. Significant main effects for both restraint, F(1,47) = 22.1, p = 0.0001, and copper deficiency, F(1,47) = 8.2, p = 0.0063, were found. There were no significant interactions at any time. Group 4, which was both copper deficient and restrained, had the highest mean BP (131 mmHg) while Group 1, the control group, had the lowest mean BP (114 mmHg). Rats adequate in copper and restrained (Group 2) and those deficient but unrestrained (Group 3) had BPs between the other groups.

Premature deaths were not evenly distributed among the groups. Only 5 (of 15) rats survived among those rats (Group 4) subjected to both deficiency and restraint. One rat died in Group 2. Although there was no statistical interaction between restraint and deficiency for BP, the high mortality in Group 4 suggests a biological interaction. Hemorrhage was prominent among the restrained, deficient rats; blood was found in the bladder, gut and peritoneum (two rats each) and in the scrotum (one rat); these findings may be due to a weakened vascular system insufficiently resistant to increased blood pressure. Similar hemorrhages (unpublished) have been found occasionally in our experiments.

Metallic elements were measured in several organs of all surviving rats. As expected (2, 13, 20, 34, 40, 41, 46), copper deficiency significantly reduced concentration of copper in all organs (Table 3). The smallest significant decrease was that in brain, 9%, F(1,44) = 4.7, p = 0.035. Decreases in other organs ranged from 29% in heart to 59% in adrenal. Copper deficiency increased cardiac sodium by 6%, F(1,44) = 5.7, p < 0.025, but had no significant effect on sodium in other organs. Potassium was decreased 2.6% in heart, F(1,44) = 4.3, p < 0.05, by restraint and was increased 5% in kidney by deficiency, F(1,44) = 4.4, p < 0.05. The only significant effect of restraint on any metallic element in any organ was this decrease in cardiac potassium. No significant differences in cholesterol in plasma or in heart or adrenal weight were found (Table 4).

EXPERIMENT 2

Because of the high mortality in the group deficient in copper and subjected to restraint in Experiment 1, we decided to increase the size of this group. It was hoped that sufficient numbers of animals would survive the experiment to protect against any statistical bias resulting from the deaths of the weaker, or more susceptible animals.

METHOD

Animals, analyses, apparatuses, diets and procedures were similar to those of the first experiment. Initial matching was into 5 groups of 15 with a mean weight of 48 g. When the rats reached a mean weight of 275 g, blood pressure was measured. Then copper was omitted from the drinking solution of the three groups with the lowest mean blood pressure and the groups with the two lowest mean blood pressure were combined into one group of 28. A small sample of hair was shaved from the backs of the animals and was discarded. At the end of the experiment, hair was obtained from these areas for analysis. Aortas and other organs were obtained as in the first experiment. Restraint was continued one week longer in Experiment 2 than in Experiment 1.

RESULTS AND DISCUSSION

Analysis of variance of blood pressure was computed across

TABLE 4
MEAN WEIGHT OF BODY AND ADRENAL GLAND, CONCENTRATION OF CHOLESTEROL IN PLASMA AND HEART WEIGHT

<table>
<thead>
<tr>
<th></th>
<th>Body (g)</th>
<th>Adrenal Gland (mg)</th>
<th>Cholesterol (mg/dl)</th>
<th>Heart (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: CuS, NR</td>
<td>367 ± 6.5</td>
<td>50.4 ± 3.2</td>
<td>114 ± 8.6</td>
<td>1.24 ± 0.03</td>
</tr>
<tr>
<td>Group 2: CuS, R</td>
<td>357 ± 7.2</td>
<td>50.8 ± 1.5</td>
<td>104 ± 2.8</td>
<td>1.21 ± 0.02</td>
</tr>
<tr>
<td>Group 3: CuD, NR</td>
<td>333 ± 5.7</td>
<td>49.3 ± 2.4</td>
<td>143 ± 14.4</td>
<td>1.30 ± 0.04</td>
</tr>
<tr>
<td>Group 4: CuD, R</td>
<td>337 ± 9.8</td>
<td>58.9 ± 4.7</td>
<td>115 ± 6.9</td>
<td>1.28 ± 0.06</td>
</tr>
</tbody>
</table>

Mean ± S.E.; CuS = copper supplemented, CuD = copper deficient, NR = nonrestrained, R = restrained.
TABLE 5
BLOOD PRESSURES (mmHg)

<table>
<thead>
<tr>
<th></th>
<th>Week of Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Group 1: CuS, NR</td>
<td>120 ± 4.3 (14)</td>
</tr>
<tr>
<td>Group 2: CuS, R</td>
<td>117 ± 4.6 (15)</td>
</tr>
<tr>
<td>Group 3: CuD, NR</td>
<td>116 ± 3.8 (15)</td>
</tr>
<tr>
<td>Group 4: CuD, R</td>
<td>115 ± 3.5 (28)</td>
</tr>
</tbody>
</table>

Mean ± S.E.; CuS = copper supplemented, CuD = copper deficient, NR = non-restrained, R = restrained; (n) = number of surviving rats.

The table shows blood pressures (in mmHg) for different groups of rats under varying conditions of restraint. The data are presented for weeks 0 through 4. The only significant main effect was for time, F(4,61) = 25.5, p = 0.0001. There were significant interactions between copper status and time, F(4,61) = 3.4, p = 0.015, and between restraint and time, F(4,61) = 8.3, p = 0.0001, indicating that it takes time before either restraint or deficiency can affect blood pressure. None of the other interactions were significant. Analysis of variance computed in week four revealed both copper deficiency, F(1,63) = 9.8, p < 0.003, and restraint, F(1,63) = 8.2, p = 0.006, significantly elevated BP. There was no significant interaction. As in Experiment 1, the highest mean blood pressure was in the restrained, deficient group and the lowest mean was in the non-restrained, supplemented group.

TABLE 6
COPPER IN ORGANS, µg/g, DRY WEIGHT

<table>
<thead>
<tr>
<th></th>
<th>Adrenal Gland</th>
<th>Brain</th>
<th>Hair</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: CuS, NR</td>
<td>1.34 ± 0.09</td>
<td>8.3</td>
<td>0.16</td>
<td>10.4</td>
<td>1.10</td>
<td>16.7</td>
</tr>
<tr>
<td>Group 2: CuS, R</td>
<td>1.73 ± 0.18</td>
<td>8.6</td>
<td>0.13</td>
<td>10.4</td>
<td>0.75</td>
<td>17.1</td>
</tr>
<tr>
<td>Group 3: CuD, NR</td>
<td>0.55 ± 0.07</td>
<td>7.4</td>
<td>0.14</td>
<td>4.7</td>
<td>1.37</td>
<td>7.3</td>
</tr>
<tr>
<td>Group 4: CuD, R</td>
<td>0.51 ± 0.04</td>
<td>7.5</td>
<td>0.10</td>
<td>5.2</td>
<td>0.59</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Mean ± S.E.; CuS = copper sufficient, CuD = copper deficient, NR = non-restrained, R = restrained.
ment, and the decrease in liver zinc due to deficiency in the second experiment. We generally (2,32) but not invariably (20) have found a decrease in liver zinc in copper deficiency.

**GENERAL DISCUSSION**

Increased mortality and hemorrhage in deficient animals subjected to stress is not surprising. Blood vessels of poor quality (51) due to defective connective tissue (26,46) from deficiency may leak when blood pressure is elevated due to stress. Synthesis of some connective tissue in arteries is dependent on lysyl oxidase, a copper metalloenzyme (43). Furthermore, copper deficiency impairs the coagulability of blood (38) which could contribute to the leakage.

The greater mortality in the deficient group subjected to stress in Experiment 1 in comparison to Experiment 2 cannot be explained by differences in diet because dietary copper was very similar between the experiments (over-all mean 0.98 mg copper per kg of diet). Groups of rats obtained at different times probably vary somewhat in the ability to resist the effects of the regimen. Even within a group of rats fed the diet in a study of mortality in which 75% were dead from deficiency in 73 days, those remaining lived 40 to 415 days longer (Klevay, unpublished data).

Cardiac enlargement from copper deficiency has been found numerous times (2, 13, 22, 50). Enlargement was found in both experiments (p<0.0002) if heart weight was expressed as a percent of body weight which corrects for the smaller size of deficient rats. Failure to find enlarged hearts in Experiment 1 without this correction may have been due to the low number of hearts available for study due to mortality. Enlarged hearts often are associated with hypertension (52) or other heart damage.

Rats subjected to two insults may be more vulnerable than rats subjected to only one (29). Moore et al. (40) found that mortality was more frequent and occurred earlier when rats were both deficient in copper and were fed extra salt. The increase in blood pressure occurred earlier in the present experiments than in similar experiments involving only copper deficiency in which no mortality occurred as of 86 days when mean blood pressure due to deficiency was 158 ± 2.4 mm of mercury (27). The rats in this experiment and the others (27,40) were all of the Sprague-Dawley strain which is not known to be susceptible to hypertension and is thought to be protected against the hypertensive effects of stress (10,36).

Ischemic heart disease is one of the major sequelae of hypertension in humans (19). Some of the effects of stress on this illness are indirect (e.g., via hypertension), others are direct. Copper deficiency, copper metabolism, hypertension and ischemic heart disease are associated (vide supra) (26, 30, 31, 37).

In summary, blood pressure and mortality were increased in adult rats both by stress and by copper deficiency. The effects on blood pressure were independent; the effects on mortality appeared to be synergistic. Results are germane to the pathophysiology of ischemic heart disease because stress is a prime factor in the induction of human hypertension, and hypertension is a risk factor for ischemic heart disease. Environmental stress and human diets low in copper may contribute to this illness.

**REFERENCES**

7. Chipperfield, B.; Chipperfield, J. R. Discussion in meat content of the heart muscle in death from ischemic heart disease. Am. Heart

95:733; 1978.
14. Harris, A. H.; Gilliam, W. J.; Findley, J. D.; Brady, J. V. Instrumental conditioning of large magnitude daily, 12-hour blood pres-