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The Influence of High Concentrations of Dietary Copper on Vitamin K-Dependent Coagulation Factors (41461)

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Abstract. It has been shown that high concentrations of some copper complexes can inhibit *in vitro* the vitamin K-dependent carboxylation of specific glutamic residues. The present study was conducted in order to determine whether high levels of dietary copper would affect the circulating levels of blood clotting factors. Rats fed for 7 weeks purified diets containing 10 (control), 100, 1000, or 2000 μg/g Cu had liver copper concentrations of 4, 4, 67, and 603 μg/g Cu, respectively. The severity of the toxicity of the 2000 μg/g Cu diet was evidenced by four deaths in this diet group. Despite the severity of the copper toxicity, values for one-stage prothrombin, activated partial thromboplastin, and Russell's Viper Venom times were similar for all groups. In addition, dietary copper level had no effect on the circulating levels of the specific vitamin K-dependent coagulation factors II, VII, IX, and X. These data provide evidence that an impairment in the blood clotting cascade is not a characteristic of dietary copper toxicosis.

The acute, or chronic, excess ingestion of copper can be accompanied by several signs including vomiting, epigastric pain, diarrhea, jaundice, hemaglobinuria, hematuria, oliguria, hemolytic anemia, and death (1). It has recently been suggested that an additional lesion of copper toxicosis may be an impairment in the blood clotting cascade (2). This suggestion was based on the observation that copper, either as a simple salt such as copper sulfate, or in the form of some complexes, such as copper aspirinate, copper penicillamine, or copper tyrosine, can inhibit the *in vitro* synthesis of prothrombin in a rat liver microsomal fraction (2–4). The inhibitory action of the copper complexes is believed to be the result of their ability to act as superoxide scavengers. The superoxide ion and/or peroxide and resultant reactants are thought to be directly involved in the vitamin K-dependent carboxylation step as they are involved in the production of the carboxylating species, perhaps through formation of the peroxybicarbonate anion or the peroxybicarbamate anion (2). In this paper we present data on the effects of high dietary copper on the activity of vitamin K-dependent coagulation factors in blood plasma of rats.

Materials and Methods. Female Sprague–Dawley rats weighing 130 ± 10 g were obtained from a commercial source (Simonsen Laboratories, Gilroy, Calif.) They were housed individually in stainless steel cages in a temperature (23°) and light-controlled room (12 hr light/12 hr dark, starting at 0800) and were acclimated for one week to a control purified diet (5) containing 10 ppm copper as copper sulfate. After the first week the rats were assigned to one of five groups: group I received the purified control (10 μg/g copper) diet; groups II, III, and IV received the same purified diet as group I with the exception that it was supplemented with copper sulfate to levels of 100, 1000, and 2000 μg/g copper, respectively; group V received the same purified control (10 μg/g copper) diet as group I, and in addition, received daily intraperitoneal injections of 450 μg of copper chloride/100 g body weight. Diets and distilled water were provided *ad libitum* throughout the experimental period.

The experiment was terminated after 7

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weeks. The animals were anesthetized with ether and opened along the ventral midline, exposing the heart and liver. Blood (2.7 ml) was collected by cardiac puncture using plastic syringes containing 0.3 ml of 3.8% citrate. A liver sample was taken from each animal and stored frozen in an acid-washed snap-cap plastic vial until time of analysis.

The blood was centrifuged in plastic tubes at 3000*g* for 10 min. Plasma was collected with plastic pipets and stored at −70° in snap-cap plastic vials until time of analysis.

The coagulation screening tests included determination of one-stage prothrombin time (PT), activated partial thromboplastin time (APTT), and Russell's Viper Venom time (RVVT). The PT assays the extrinsic and common coagulation pathways which includes factors VII, X, V, prothrombin (II), and fibrinogen (I). The APTT assays the intrinsic and common coagulation pathways which includes factors XII, XI, IX, VIII, X, V, II, and I. The RVVT assays the common coagulation pathway which includes factors X, V, II, and I. This trio of tests is performed as a panel to delineate specific areas of coagulation protein deficit. All of these tests were performed according to previously described methods (6) using semiautomated equipment.[2]

Assays of factors II, VII, IX, and X were performed with semiautomated equipment[2] by previously described methods (7).

Pooled serum from 25 Sprague–Dawley rats was used to prepare standard curves. The results for test animals were compared with the curve and expressed as percentage of normal.

Liver samples were wet ashed with 16 *N* nitric acid, concentrated by evaporation, and diluted with distilled deionized water. Copper concentrations were determined by atomic absorption spectrophotometry (Perkin–Elmer Model 370, Norwalk, Conn.) (8).

Results and Discussion. The average weight of rats in group I (control) at the termination of the experiment was 214 ± 15 g. Group II (100 μg/g CU), group III (1000 μg/g Cu), and group V (IP Cu) rats had terminal weights (196 ± 17, 198 ± 11, and 191 ± 16 g, respectively) which were similar to control values, while group IV (2000 μg/g Cu) weighed significantly less ($P \leq 0.05$) than controls (167 ± 26 g). The finding of growth retardation with the development of copper toxicosis is consistent with the observations of previous investigators (9, 10). The observed growth retardation has been correlated with reduced food intake. Indeed, rats consuming a diet containing 4000 μg/g copper were reported to restrict food intake so severely that partial starvation, and death occurred within one week of initiation of the diet. Interestingly, due to the low food intake, the actual amount of copper received by group IV animals in the present study was similar to that of animals consuming a diet containing only 500 μg/g copper; thus, the reduction in intake may be due to the taste of the diet and/or to postingestional consequences such as nausea or other malaise, and not to a direct systemic action of the element (9).

A conditioned taste aversion to, and avoidance of a diet occurs when consumption of that diet is followed by illness (11). It is reasonable to assume that the 2000 μg/g copper-containing diet consumed by group IV produced unpleasant gastrointestinal consequences, resulting in their subsequent avoidance of the diet, reflected in reduced food intake. The mechanism of conditioned taste aversion could also have contributed to a slightly reduced food intake observed in group V, which received acceptable (control) levels of copper in the diet, yet received excess copper through intraperitoneal administration. Injection of an illness-producing agent paired temporally with consumption of a harmless substance can produce a taste aversion to, and avoidance of that substance, even though the compound itself does not cause the illness (12). In addition, the copper-injected rats may have "tasted" the copper through intravascular stimulation of the gustatory receptors. The rat gustatory nerve responds to intravascularly injected compounds, and taste aversions are readily conditioned to

[2] Fibrosystem, Bioquest Div., Becton, Dickinson and Co., Cockeysville, Md.

an innocuous tastant introduced intravascularly, providing that its taste is paired with illness produced by consumption of, or intraperitoneal injection of a toxic agent (13).

The animals in groups I, II, and III looked healthy throughout the experimental period, although a few of the rats in group III developed a minor degree of diarrhea toward the end of the experiment. In contrast, the majority of the animals in groups IV and V developed diarrhea by the third week of the experiment, which then persisted throughout the remainder of the experiment. Animals in groups IV and V were listless in comparison to animals in groups I–III. The severity of the copper toxicosis induced by the feeding of the 2000 μg/g copper diet (group IV) was evidenced by four deaths in this group, occurring on Days 17, 35, 38, and 47 of the experiment. Deaths did not occur in any of the other groups. Because of the rapid deterioration of the remaining animals in group IV, the experiment was terminated on Day 47, 2 days short of the completion of the seventh week.

No gross pathological lesions were observed in animals from groups I–III. In contrast it was observed that several of the rats in group IV had enlarged, very dark spleens, and very dark, hemoglobin-stained kidneys. Similar observations were made for rats in group V, which in addition had a considerable number of abscesses in the liver and peritoneal cavity. Cellulitis and abscess formation found at the sites of subcutaneous injection of copper have been suggested to be the result of acute local copper toxicosis (14). The enlarged and discolored spleens were presumably due to hemolytic anemia, a common occurrence in copper toxicosis (15).

Liver copper concentration was similar in rats from groups I and II ($\bar{x}$ = 4.3, 4.5 μg/g, Table I), while rats in groups III–V had liver copper values which were significantly greater ($P \leq 0.01$) than the group I and group II values (66.8, 602.6, 218.6 μg/g, respectively). Thus the highest liver copper values were found for the 2000 μg/g copper-fed animals, the only group in which deaths occurred. The liver copper concentration of group IV was similar to that reported by previous investigators who fed similar levels of copper (9). The second highest concentration of liver copper was found in the copper-injected animals, a group observed to have gross pathological lesions at the time of killing.

There were no significant differences among the groups for any of the screening clotting tests studied (Table I) or for vitamin K-dependent factors (Table II). While it should be noted that there was considerable variation in liver copper values within the copper-loaded groups, as evidenced by the large standard errors, there was no correlation between liver copper concentration and screening clotting test time within any single group.

The function of the normal hemostatic mechanism is to prevent blood loss from

TABLE I. THE EFFECT OF HIGH CONCENTRATIONS OF DIETARY COPPER ON PLASMA COAGULATION SCREENING TESTS[a]

Group	Diet	N	Liver Cu[b]	P T[c,d]	APTT[e]	RVVT[f]
I	Purified diet (10 ppm Cu)	13	4.3 ± 0.2	14.34 ± 0.76	54.77 ± 1.65	13.56 ± 0.69
II	Purified diet (100 ppm Cu)	13	4.5 ± 0.1	14.22 ± 0.16	50.95 ± 2.79	16.69 ± 0.72
III	Purified diet (1000 ppm Cu)	14	66.8 ± 23.5*	13.83 ± 0.33	51.51 ± 1.58	15.39 ± 0.46
IV	Purified diet (2000 ppm Cu)	10	602.6 ± 131.3*	14.19 ± 0.38	54.74 ± 3.29	15.22 ± 0.76
V	Purified diet (10 ppm Cu) + ip injections of 450 μg of $CuCl_2$/100 g body wt daily.	15	218.6 ± 18.4*	15.50 ± 0.19	47.15 ± 3.28	18.11 ± 0.72

[a] All values are expressed as mean ± SEM.
[b] In μg/g tissue wet weight.
[c] Seconds.
[d] One-stage prothrombin time.
[e] Activated partial thromboplastin time.
[f] Russell's Viper Venom time.
* Significantly greater than control values (group 1) by $P \leq 0.01$ using Student's t test.

TABLE II. THE EFFECT OF HIGH CONCENTRATIONS OF DIETARY COPPER ON VITAMIN K-DEPENDENT COAGULATION FACTORS[a]

Group	N	Factor II	Factor VII	Factor IX	Factor X
I	13	92 ± 6	105 ± 7	78 ± 8	103 ± 11
II	13	91 ± 9	98 ± 9	84 ± 11	91 ± 9
III	14	88 ± 4	93 ± 4	89 ± 6	111 ± 8
IV	10	101 ± 11	107 ± 12	91 ± 6	96 ± 7
V	15	87 ± 11	91 ± 8	99 ± 7	88 ± 6

[a] Expressed as a percentage of a plasma pool (25 individuals) of female Sprague–Dawley rats considered to be normal individuals. The pooled plasma was assumed to contain 100% of each factor assayed. All values expressed as mean ± SEM.

intact blood vessels and to stop excessive bleeding from injured vessels. Arrest of bleeding is controlled by three interrelated events. These three events are the reaction of blood vessels to injury, the formation of the platelet plug at the site of injury, and the coagulation of blood. The process of blood coagulation occurs in a series of complex steps which terminates in the formation of a fibrin clot. Blood coagulation occurs either by activation of the intrinsic pathway, or the extrinsic pathway. The intrinsic pathway is within the blood vascular system, while the coagulation in the extrinsic pathway is initiated by a factor extrinsic to the blood vascular system.

Vitamin K is an essential cofactor for the carboxylation of specific glutamic acid residues on coagulation factor proteins II (prothrombin), VII (proconvertin) IX (Christmas factor; Hemophilia B factor), and X (Stuart–Prower factor). Vitamin K completes the final step in the biosynthesis of these coagulation factors by converting glutamic acid residues on precursor molecules to γ-carboxyglutamic acid residues, producing functionally normal factors. Factor VII is involved in the extrinsic pathway and has the shortest half-life of all the coagulation proteins. Factor IX is involved in the intrinsic pathway and has the shortest half-life of the coagulation proteins excepting factor VII. Factors II and X are involved in the common pathway; this coagulation pathway is common to both the intrinsic and extrinsic pathways.

The results obtained in this study show that clotting dysfunction is probably not a clinical complication in copper toxicosis. However, it should be stressed that these data do not contradict the findings of Esnouf and his colleagues on the inhibitory action of copper-containing superoxide scavengers on the vitamin K carboxylation step in isolated liver microsomes. The inability to detect changes in the circulating factor coagulation times may be a consequence of the following: (i) the carboxylase system *in vivo* is not inhibited to a rate-limiting degree, and/or (ii) liver copper metabolism *in vivo* precludes a high microsomal concentration of copper complexes having superoxide dismutase activity.

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