Decreasing Ethanol Consumption in Ethanol-Dependent Rats through Supplementation of Zinc and Copper Amino Acid Chelates: A Preliminary Study

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ABSTRACT

Objective: Ethanol abuse exacerbates copper (Cu) and zinc (Zn) deficiencies resulting in metabolic alterations that frequently lead to increased ethanol intake. This preliminary study examined the effect of Cu and Zn amino acid chelate (AAC) supplementation on ethanol intake in ethanol-dependent rats compared to non-supplemented ethanol-dependent rats.

Methods: After creating ethanol dependency in two groups of 10 rats each, the animals in the treated group were each gavaged with 100 µg Zn and 45 µg Cu daily as AAC for 21 consecutive days. All animals in both groups had free access to water and also to a 5% ethanol/95% water mixture (v/v) throughout the study period. Measurements included voluntary consumption of ethanol/water and of water and changes in weights. Pathological and histopathological examinations and Cu and Zn analysis of certain tissues were also done.

Results: While both groups of animals exhibited initial behavior suggesting ethanol dependency, which was subsequently confirmed by pathological examinations of their tissues, the group receiving Cu and Zn AAC voluntarily began restricting its ethanol/water intake ($P<0.001$) and increasing its water consumption ($P<0.001$) compared to non-supplemented control animals.

Conclusions: Oral supplement of copper and zinc amino acid chelates appeared to reduce the physical dependency for ethanol.

INTRODUCTION

When ingested in small quantities, ethanol is utilized as a source of energy through its oxidation to acetaldehyde by zinc $(Zn)^{2+}$ activated alcohol dehydrogenase (ADH) followed by further oxida-

tion of the acetaldehyde to acetic acid.[1] When ADH activity is overwhelmed by excessive ethanol, cytochrome P-450 (cyP_{450}) activity increases with superoxide radicals being the primary resultant metabolite. This leads to increased lipid peroxidation in the brain, prostate, liver, nerve cells, and blood (ceruloplasmin) coupled with decreased copper (CU)/Zn superoxide dismutase (SOD) activity.[1-7] Increased superoxide radical tissue concentrations have been reported to result in greater physical dependency for ethanol.[8] SOD activity tends to block cyP_{450} activity and prevent superoxide radical accumulation in the cytoplasm.[9]

Because of Zn's role in ADH, coupled with its further involvement in SOD, there is a substantial requirement for hepatic Zn. Chronic and excessive intake of ethanol long term tends to produce Zn deficiencies in the liver and other tissues by interfering with its absorption and metabolism with concurrent increases in urinary and fecal Zn excretion of Zn.[10,11] When compared to controls, rats consuming ethanol daily for 30 days had significantly lower hepatic Zn concentrations.[11-14]

Cu is the activating co-factor of SOD.[15] Consumption of ethanol reduces hepatic Cu concentrations resulting in lower SOD and glutathione peroxidase activity.[16,17] Ethanol ingestion also has been reported to interfere with Cu metabolism.[18]

When Zn and Cu are chelated to amino acids (AAC), the absorption rates of both metals are significantly greater than those of equivalent amounts of the same metals as inorganic salts. Mucosal absorption of Cu from AAC has been reported to be 4.4 times greater than from $CuSO_4$.[19] When Cu was administered concurrently with the dietary Cu antagonists—molybdenum, sulfur and iron—experimental animals receiving Cu AAC maintained higher Cu liver concentrations than similar animals receiving other Cu sources ($P<0.05$).[20]

Mucosal absorption of Zn from AAC has been reported to be 2.2 times greater than from $ZnSO_4$.[19] In experimental animals receiving a single dose of ^{65}Zn AAC there was a mean 19% increase of ^{65}Zn in the tissues ($P<0.05$) coupled with a 36% increase in hepatic ^{65}Zn ($P<0.05$) compared to ^{65}Zn from $^{65}ZnCl_2$.[19]

SOD activity has been reported to increase following oral administration of Cu and Zn AAC. In animals receiving equivalent amounts of Cu and Zn as AAC or sulfate for 64 days, erythrocyte SOD activity increased 11.7% in the AAC group compared to a 3.79% decrease in the sulfate group ($P<0.05$).[21] In a clinical study involving 23 individuals with rheumatoid arthritis (RA), each received 2 mg Cu/d as AAC for 21 days. Erythrocyte SOD activity increased 21% ($P<0.001$) in the RA group and was equivalent to matched subjects not afflicted with RA.[22]

Since ethanol consumption depletes tissue levels of Cu and Zn with a corresponding reduction in SOD activity and potentially creates increased physical ethanol dependency, we hypothesized that, due to their greater bioavailability with consequential increased SOD activity, Cu and Zn AAC supplementation may be able to help overcome ethanol dependency associated with ethanol toxicity. We, therefore, designed a study to test this hypothesis.

MATERIALS AND METHODS

Twenty male Sprague-Dawley albino rats about 2 months of age and each weighing approximately 190 ± 22.9 g were divided into two groups of 10 each and randomly assigned to either a control or treatment group. There were no significant differences in measured parameters (weight and age) between the animals ($P>0.05$). The rats were housed individually in stainless steel

cages. The temperature in the facility was maintained at approximately 20°C. Food consisted of a commercial dry laboratory rat chow, which was fed *ad libitum*. This basal diet (AIN 76 A diet) contained 20 ppm of Zn ($ZnCO_3$) and 15 ppm of Cu ($CuCO_3$).

Each cage was fitted with two appropriately labeled bottles, one containing water and the other containing a mixture of 5% ethanol and 95% water (v/v). All water used in the study was distilled and deionized. The positions of water and ethanol/water mixture bottles were changed daily to reduce likelihood that learning bottle locations would influence selection of either ethanol or water. Both water and the ethanol/water mixture were available throughout the entire study to each rat *ad libitum*.

Liubimov et al reported that forced daily administration of 8 g (10 mL) ethanol/kg per day (2/3 of the LD-50) to rats for 28 days produced ethanol dependency.[23] Since ethanol metabolism is greater in rats than humans, a higher sustained blood alcohol concentration is required to induce dependency.[17] At study commencement, each animal in both groups was weighed and subsequently administered approximately 1.52 g (1.9 mL) of ethanol per day for the first 7 days by gastric gavage using a syringe with a curved balled needle. The ethanol was diluted with distilled and deionized water so that the final solution (3.8 mL) gavaged into the stomach of each rat contained 50% ethanol and 50% water by volume. Each rat continued to be weighed weekly on an electronic balance and the daily quantity of administered ethanol adjusted according to each rat's individual weekly weight so as to maintain the forced administration of approximately 8 g ethanol/kg per day. Administration occurred at approximately the same time daily for 28 days. Including the ethanol gavaged into the rats, actual 24-hour intake of ethanol ranged between 8 and 10 g/kg per day per animal by the 28th day of gavage.

Forced administration of ethanol was discontinued on day 28. On day 29 each animal had to acquire all desired ethanol from its ethanol-dispensing bottle attached to the cage. Consumption of both the ethanol/water mixture and water was measured each 24 hour period.

On day 29 each rat in the treated group began receiving Zn AAC and Cu AAC daily. Both chelates were dissolved in distilled and deionized water. Each dose contained 100 µg Zn (approximately 0.49 mg/kg) and 45 µg Cu (approximately 0.22 mg/kg). This mineral solution was administered intragastrically at approximately the same time daily for days 29 through 49 to the treated group using the same syringe and curved balled needle apparatus described above.

Although the control group received dietary Cu and Zn in its food, it did not receive supplemental Cu or Zn during the study.

On day 50 of the study, all animals were killed using halothane as an anesthetic followed by cardiac puncture for exsanguination. Terminal body weights were recorded prior to exsanguination.

The brain, spinal cord, thymus, trachea, esophagus, cervical lymph nodes, heart, kidneys, urinary bladder, skeletal muscle, skin, lungs, thyroid glands, adrenal glands, stomach, duodenum, jejunum, ileum, cecum, colon, pancreas, spleen, testes, and liver of each animal in the study were subjected to a gross pathological examination. Further, each animal's brain, heart, kidneys, skeletal muscle, adrenal glands, stomach, duodenum, jejunum, ileum, cecum, colon, pancreas, and liver were studied histopathologically. Following histopathological sampling, each animal's brain, liver, kidneys, and stomach were individually assayed for Cu and Zn

concentrations by ICP (inductively coupled plasma emission spectrophotometry).[24,25]

During this study, all animals were housed in the life science laboratories of Weber State University, Ogden, Utah, and handled according to the university protocol relating to laboratory animals. The study protocol was approved by the ethical committee of the university prior to commencement.

All data collected in this study were analyzed statistically using Systat Version 10 (Systat Software Inc., Richmond, CA). The Student's *t*-test was used to determine statistical significance. An alpha level of 0.05 or less was set for determining statistical significance. If the alpha level was between 0.05 and 0.10, it was considered trending toward significance.

RESULTS

By the end of the initial 28-day period, all rats in both groups exhibited behavior suggesting ethanol dependency. Twenty-four hour ethanol intake ranged from 8 to 10 g/kg per day per animal in the latter part of the initial 28-day period with a portion being administered by gastric gavage and the rest acquired from the ethanol/water bottle. By day 28 both groups appeared to prefer the ethanol/water mixture to water alone. As a percent of total fluid intake, water consumption decreased in both groups during the first 28 days with corresponding increases in ethanol/water consumption. There were no significant differences between groups in either water or ethanol/water consumption during the first 28 days of the study.

Gross pathology indicated gastrohepatic and intestinal-peritoneal adhesions consistent with ethanol abuse in all animals. Additionally, one rat from each group had developed upper mesenterichepatic adhesions. Histopathology examinations revealed inflammation of both the mucosal and serosal surfaces of the intestines. Livers contained focal and periportal abscesses as well as general and focal inflammation. All lesions and inflammation in both groups were graded minimal to mild, but were of the types generally associated with chronic ethanol toxicity. The remaining tissues appeared to be normal.

The kidneys from the control group contained significantly ($P<0.05$) more Zn (45.45 ppm vs. 21.2 ppm, wet tissue) and significantly ($P<0.05$) more Cu (9.3 ppm vs. 8.5 ppm, wet tissue) than kidneys of the treated group. Zn and Cu analysis of the brains showed trends (Zn: $P=0.09$; Cu: $P=0.082$) toward greater amounts of these metals in the brains of the treated group, (Zn: 15.3 vs. 10.4 ppm, wet tissue; Cu: 8.2 vs. 6.9 ppm, wet tissue). There were also trends toward greater increased amounts of Zn ($P=0.08$; 41.1 vs. 39.8 ppm, wet tissue) and Cu ($P=0.073$; 9.4 vs. 7.7 ppm, wet tissue) in the livers of the treated group. There were no differences in the Cu and Zn levels in stomach tissues between groups.

Figure 1 summarizes mean water consumption per rat per day and includes mean trend lines for both groups beginning on day 29. Measurement commenced at 9 a.m. on day 29 when each water bottle was filled to a pre-determined level. On day 30 at 9 a.m., when the bottle was replenished, water consumption for the first 24-hour period was recorded, and so on.

Following administration of Cu and Zn AAC, the mean quantity of water consumed per day from day 29 to day 49 by the treated group was 2.6 times greater than that of the control group ($P<0.001$). Based on the trend lines in Figure 1, both groups of animals increased their water consumption over time similarly, but as noted above, the treated group consumed more water per day than the control group.

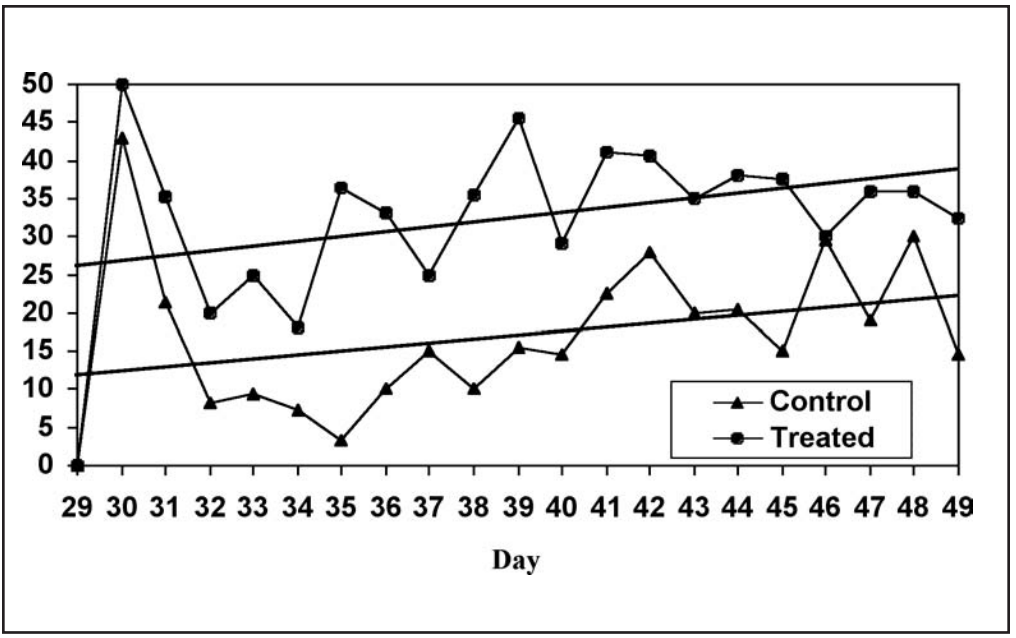

Figure 1. Mean daily water consumption from day 29 to 49 in treated and control rats. Rats supplemented with zinc (Zn) and copper (Cu) amino acid chelate (AAC) (treated group) consumed more water per day than non-supplemented rats (control group). Differences were significant (P<0.001).

Figure 2 shows the mean consumption of the ethanol/water mixture per rat per day from day 29 to day 49. It also shows mean ethanol/water consumption trends for both groups. The procedure for measuring consumption was similar to that of the water. The mean daily consumption of the ethanol/water mixture was 11.35 times greater for the control group than for the treated group (P<0.001). While the ethanol intake by the treated group remained relatively constant from day 30 through 49, as indicated by the flat trend line for that group, to the contrary, there was a trend toward increased voluntary ethanol/water consumption by the control group.

Water to ethanol/water mixture ratios were calculated for each group. From day 29 to day 49, approximately 2.5% of the treated group's total liquid intake was derived from the ethanol/water mixture compared to approximately 61.0% for the control group (P<0.001).

When mean water and ethanol/water consumptions were combined, the mean daily liquid consumption per animal in the treated group from day 29 to day 49 was 34.8 ± 7.8 mL with about 0.1 mL of that total amount being contributed by ethanol (0.3% of the total). In the control group, the mean consumption of total liquid per day was 24.3 ± 10.3 mL with ethanol contributing about 2.3 mL of the total liquid (9.5%). The difference in total liquid consumption was significance (P<0.01). Even though its total liquid consumption was less, from day 29 to 49 the control group's average consumption of ethanol was 22.8 times greater than that of the treated group (P<0.001). These data are summarized in Table 1.

The mean initial weight of the control group was 191.1 ± 21.8 g and the treated group, 189.6 ±24.0 g. The mean terminal weight of the control group was 233.8 ± 24.3 g compared to 210.9 ± 13.8

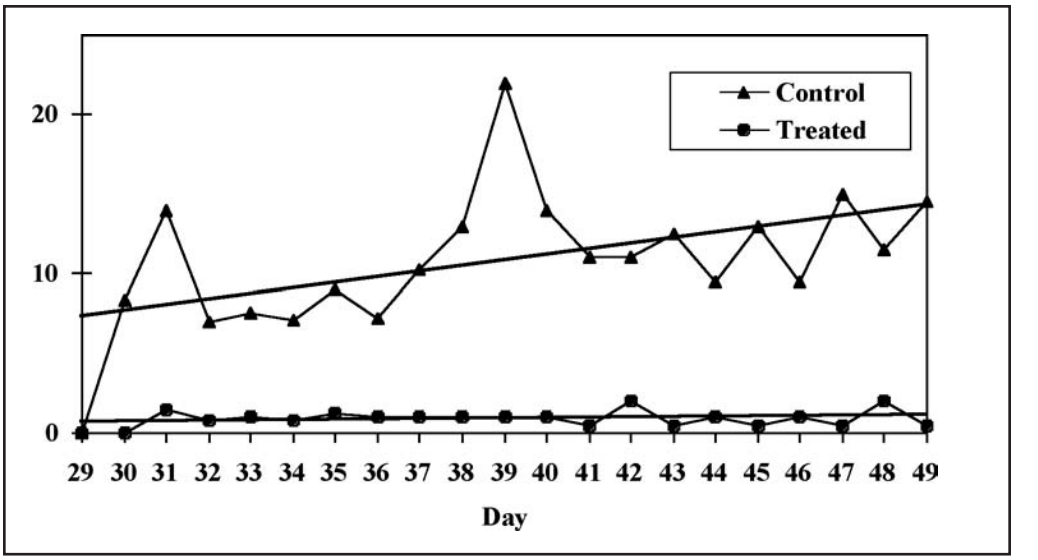

Figure 2. Mean daily ethanol/water mixture consumption from day 29 to 49. Non-supplemented rats (control group) voluntarily consumed approximately 11.35 times more ethanol/water per day ($P<0.001$) than did Zn and Cu AAC supplemented rats (treated group).

g for the treated group ($P<0.05$). These data are summarized in Table 1.

DISCUSSION

When forced ethanol administration was terminated on day 28 of the study, both groups had to seek desired ethanol from ethanol/water bottles. Initial daily voluntary ethanol consumption from day 0 to day 28 was similar for both groups, but by day 30 (24 hours after the treated group received its first dose of Cu and Zn AAC) there was distinct difference between groups. The control group continued favoring the ethanol/water mixture, whereas the treated group began favoring water. This is clearly seen in Figures 1 and 2, particularly when mean trend lines are compared. The slope of the trend line of the treated group for ethanol consumption is flat (Figure 2) coupled with greater total water consumption (Figure 1), whereas the slope of the trend line for ethanol consumption in the control group increased over time (Figure 2) with lower total water consumption (Figure 1). While not measured in this study, the greater intake of ethanol by the control group suggests, as the literature has reported, that the ethanol may be overwhelming normal ADH activity resulting in increased accumulation of superoxide radicals in the tissue. As reported earlier, where superoxide radical tissue concentrations increase, greater ethanol consumption frequently results.

The effects of ethanol intoxication have been reported to include reduced SOD activity.[17] While no analysis of SOD activity in tissues occurred in this study, there were trends toward greater quantities of Cu and Zn in the tissues of the treated group, which previous investigators have reported leads to greater SOD activity.[21,22] The data in this study suggest that there may have been greater SOD activity in the treated group which, if true, could be postulated as a reason for that group's lower ethanol consumption following Cu and Zn AAC supplementation. The major deficiency of this study is that no SOD activity was measured in any of these animals' tissues. This should be addressed in a future study.

Table 1. Mean Weights and Liquid Consumptions in Supplemented (Treated) and Non-Supplemented (Control) Per Rat.

	Group	
	Control	**Treated**
Initial weight	191.1 ± 21.8 g	189.6 ± 24.0 g
Terminal weight*	233.8 ± 24.3 g	210.9 ± 13.8 g
Water consumption per day (days 29-49)*	12.9 ± 9.5 mL	33.9 ± 7.9 mL
Ethanol consumption per day (days 29-49)*	2.3 ± 0.72 mL	0.1 ± 0.02 mL
Ethanol/water mixture consumption per day (days 29-49)*	11.4 ± 3.6 mL	0.9 ± 0.5 mL
Total liquid consumption per day (29-49)*	24.3 ± 10.3 mL	34.8 ± 7.8 mL

Data presented as mean ± standard deviation
*Significant difference (P<0.05) between treated and control

Increased SOD activity in the liver has been reported to potentially protect that organ and, over time, reverse some of the damage resultant from chronic ethanol toxicity.[17] This study was not of sufficient duration to demonstrate that, however. The results of the hepatic histopathology examinations of both groups in this study remained similar after the 21 days of Cu and Zn AAC supplementation in the treated group. Novelli et al reported that it takes up to 300 days to return ethanol-damaged tissue to normal after discontinuing ethanol consumption.[5] It should be remembered that the treated group did not completely discontinue consumption of the ethanol (Figure 2), which could have also affected the rate of the return of the tissues to normal. It would be interesting to design a study to investigate the effects of prolonged Cu and Zn AAC supplementation on ethanol-damaged tissue.

It is important to point out that the slopes of the two trend lines for water consumption in Figure 1 are similar. While the treated group consumed more total water per day than the control group, both groups increased water consumption over time at about the same rates. One possible reason is that both groups of rats were still growing and had similar needs for increasing amounts of water as their bodies increased in size.

Tissue mineral analysis in both groups revealed more Zn in the kidneys of the control animals than the treated group suggesting greater urinary excretion of Zn in the control group, which is generally observed in cases of ethanol intoxication.[10,11] While feces is the major excretionary route for absorbed Cu, it has been reported that in cases of liver cirrhosis, urinary excretion of Cu is increased.[26] Histopathological examination of the livers suggested early cirrhosis suggesting the possibility that the increased Cu levels in kidneys of the control group reflected this phenomenon.

The higher amounts of Cu and Zn found in the kidneys of the control group compared to the treated group may also be a result of increased diuresis associated with chronic ethanol toxicity which inhibits vasopressin release from the posterior pituitary gland. This diuretic effect concurrently results in increased electrolyte excretion in the urine.[27,28] This study was not designed to collect or assay urine, but the data from renal tissue analysis appear to be consistent with published literature relating to urine analysis under similar conditions. Perhaps a future study should examine

the effect of Cu and Zn AAC on diuresis from ethanol toxicity more closely.

The control group was significantly heavier than the treated group at study termination ($P<0.05$). This observation may reflect literature reports of increased deposition of fatty tissue in animals with ethanol dependency.[18] Body fat was not measured in either group so one can only speculate as to why the control group was heavier. Perhaps a longer term study should be designed to examine weight differences in greater detail.

Whether supplementation of Cu and Zn AAC resulted in permanent changes in ethanol consumption by the treated group is unknown. During the 21 days of mineral supplementation, the treated group seemed to prefer drinking water to ethanol. Over a longer period of time, would the treated group have increased its ethanol consumption even if its diet were supplemented with Cu and Zn AAC or would ethanol abstinence have occurred? Would the animals have increased their ethanol consumption if the mineral supplementation had been discontinued? Would a Cu and/or Zn deficiency in either group of animals encouraged ethanol dependency earlier in the study? This current study raised many questions which are interesting and require additional research.

CONCLUSION

In conclusion**,** this study demonstrated that in rats suffering from ethanol dependency, supplementing the rats' diets with approximately 0.22 mg/kg per day of Cu and 0.49 mg/kg per day of Zn, both in the amino acid chelate form, resulted in a significant decrease in ethanol consumption accompanied by a significant increase in water consumption compared to non-supplemented rats. It is postulated that the higher Cu and Zn tissue levels resulting from amino acid chelate supplementation initiated increased superoxide dismutase activity in supplemented animals' tissues with a corresponding reduction in ethanol-produced free radicals. While not proven, it was postulated that this increased SOD activity was the reason for less ethanol dependency in the treated group.

DISCLOSURE

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