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Influence of Alcohol and Low Dietary Copper on Copper Utilization of Maternal and Offspring Liver

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ABSTRACT

Pregnant rats were fed liquid diet to determine the influence of maternal ethanol intake on maternal and pup liver copper when dietary copper was low. The diets, which contained either 0.75(low) or 3.75(control)mg copper/1 with or without 30% of kcal from ethanol, were fed throughout gestation and the first 15 days of lactation. Maternal calorie intake and body weight were unaffected by dietary treatment. Ethanol intake depressed maternal liver copper concentration only when diet copper was low(interactive effect $P<0.05$). Although ethanol intake depressed total pup liver copper concentration regardless of dietary copper level, the interactive effect observed in maternal liver was reflected in copper content of the pup liver metallothionein fraction eluted from a Sephadex G-75 column. The zinc content of metallothionein was inversely related to copper content of metallothionein. Results suggest that pregnancy and lactation is a special period to develop a copper deficiency when low copper intake and ethanol ingestion are combined not only in mothers but also in their offspring.

KEY WORDS : copper · alcohol · pregnancy · metallothionein.

INTRODUCTION

In contrast to what is known about the effect of alcohol on utilization of many vitamins and minerals[1], much less information is known about a copper and alcohol relationship. We do know, however, that female rats fed ethanol in their drinking water for 32 weeks cannot achieve a normal liver copper concentration relative to non-ethanol controls[2]. Furthermore, Bogden et al.[3] have demonstrated that this depressive effect of alcohol on tissue copper was specific for liver because copper content of kidney, spleen, testes, heart, brain, femur, and muscle was unaffected by ethanol in male rats after 15 weeks of exposure to ethanol in drinking water. This latter observation is important because the liver is the key organ regulating internal copper homeostasis[4]. In any case it appears that alcohol must be ingested for many months before liver copper concentration is compromised which casts doubt on the possible significance of an alcohol and copper relationship.

One situation that could significantly reduce the time required to see a depressive effect of alcohol

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on liver copper concentration would be to have a period in time that was represented by both low dietary copper intake and a high metabolic demand for copper. In human nutrition, such a situation naturally exists in the form of pregnancy and lactation. For example, current analysis of a variety of diets has found that a marginal dietary copper intake of 0.8 to 1.2mg per day[5)6)] is not unusual which is well-below the recommended daily copper intake of 1.5 to 3mg[7)]. Although men and non-pregnant women can apparently adapt to this low level of dietary copper and achieve metabolic balance[8)9)], pregnant women have difficulty in achieving copper balance even at the upper end of the provisionally recommended range of copper intake suggesting that copper requirement is greater during pregnancy[10)] than nonpregnancy in humans. A similar observation has been made in the rat[11)]. Therefore, if a marginal dietary copper intake was coupled to a period of high metabolic demand, represented by pregnancy and lactation, it may be possible to see a depressive effect of alcohol on both maternal and offspring liver copper concentration in a matter of weeks rather than months. In this regard a measurement of copper concentration in metallothionein of pup liver cytosol will be necessary to define an effect of maternal ethanol intake on pup liver copper status because this protein has been reported to play an important role in the initial uptake and temporary storage of copper in immature rat liver[12)].

The purpose of the present study was to determine if the depressive effect of alcohol on liver copper concentration would exaggerate a marginal copper status during pregnancy and lactation. Criteria of assessment included liver copper concentration in both dams and pups, and copper concentration of metallothionein in pup liver cytosol. Because of the known inverse relationship of copper and zinc[13)14)], it was also determined whether a depression in copper concentration of pup liver metallothionein led to an increase in zinc concentration in this same protein.

MATERIALS AND METHODS

Twenty-four pregnant rats(CRI : CD(SD)BR, Charles River Laboratories, Wilmington, MA) were fed individual liquid diets containing either 0.75(low) or 3.75(control)mg copper/1, with or without 30% of kcal from ethanol throughout gestation and the first 15 days of lactation. The liquid diet was designed to provide 1 kcal/ml in contrast to a solid diet which would provide an average of 4kcal/g[15)]. Therefore, 0.75mg copper/1 in the liquid diet was equivalent to a solid diet containing 3mg/kg copper or 60% of the copper recommended for growth and reproduction in the rat[15)]. Similarly, 3.75mg copper/1 of liquid diet was equivalent to a solid diet containing 15mg/kg copper. This liquid diet, which has been described in greater detail elsewhere[16)], contained(g/1) micropulverized casein, 69.75 ; DL−methionine, 0.5 ; cellulose powder, 10 ; dextrose, 144 ; corn oil, 13 ; xanthan gum, 2 ; vitamin mixture, 12.5 ; mineral mixture, 10.0 ; and sufficient distilled-deionized water to give one liter when blended. Ethanol was isocalorically substituted for dextrose so that all diets provide 1kcal/ml. The amount of liquid diet offered was restricted to the amount of liquid diet consumed per 100g body weight in the low copper plus ethanol group to minimize the effect of different dietary intake. Furthermore, rats are known to overeat liquid diets provided ad-libitum. From day 10 of gestation, body weights were taken at least twice a week to ensure adequate food intake on the basis of body weight because initial weight of rats ranged from 220 to 300g.

On day 1 of lactation, litter size for all groups was reduced to eight and dams were allowed to nurse pups to the 15th day of lactation. The study ended on day 15 of lactation to prevent consumption of diet by pups. All dams and pups were killed by decapitation while under light sodium pentobarbital anesthesia. Liver, kidney, heart and spleen from dams and pups were frozen at −60 °C.

The concentration of copper in tissues was measured by atomic absorption spectrophotometry (Perkin-Elmer Model No. 2380, Norwalk, CT). Whole liver and kidney were wet ashed with concentrated nitric acid followed by 30% hydrogen peroxide on a hot plate in a hood. The ash of tissues was dissolved in 3N HCl with gentle heat and diluted with redistilled water to an appropriate volume for analysis. Spleen and heart were dried in a vaccum oven at 60°C for 16 hours, weighed and ashed as described above.

To study the distribution of copper in the cytosolic proteins of pup liver, pooled pup livers in each treatment were homogenized with 3 volumes of 0.05M Tris-HCl buffer in isotonic saline, pH 8.6, with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 160,000×g for 90 min at 5°C. The supernatant solution (equivalent to 3g wet weight liver) was fractioned by gel filtration on Sephadex G-75 columns(2.0×100cm) was a 0.05M Tris-HCl buffer, pH 8.6, at 4°C[17]. Fractions of 4 to 5 ml were collected at a flow rate of 24 to 30ml per hour. The concentration of copper and zinc in column fractions was then analyzed by direct aspiration into an atomic absorption spectrophotometry(Model 82−516, Jarrel-Ash Co., Waltham, MA). The metal content in column fractions was calculated on the basis of 3g pooled pup livers in each treatment for gel filtration and expressed as μg/ml. Copper content of maternal liver metallothionein was not determined because this inducible protein has been reported to be barely detectable in the liver of adult rat fed low and normal concentration of zinc or copper[18].

The statistical design for this study was a 2×2 factorial experiment with six replicates per treatment[19]. Treatment effects were partitioned into effects of ethanol, copper, and the interaction of the two factors if a significant F-value was found for treatment effects. Differences between planned comparisons of means were tested by Fisher's least significant difference(FLSD). Effects were considered to be significant at $P<0.05$.

RESULTS

Energy intake and growth

Daily energy intake and body weight of dams during gestation and lactation are shown in Table 1. Daily energy intake for all dams was typical of that expected from a conventional solid diet during gestation and lactation[20]. Maternal weight gain during gestation, litter size, and average weight of 1 day-old pups were unaffected by dietary treatments. The average weight of 15 day-old pups in the low copper plus ethanol group, however, was significantly depressed when compared to pups not exposed to alcohol.

Tissue copper analysis

As shown in Table 2, the only tissue copper concentration adversely affected by ethanol in both dams and pups was the liver. In dams, liver copper concentration was only depressed by ethanol when dietary copper was low(interactive effect, $P<0.05$) whereas in pups, liver copper was decreased by ethanol regardless of diet copper level. The high copper concentration of immature pup liver compared to mature maternal liver is similar to the observation of others[21]. A similar interactive effect between maternal ethanol ingestion and diet cop-

Table 1. Influence of ethanol and copper on dietary intake and growth of dams and pup

Measures	Diet, % kcal from ethanol			
	3.75 mg Cu/l		0.75 mg Cu/l	
	0	30	0	30
Energy intake, kcal/d				
Gestation day 2	86± 11	70± 30	84± 10	81± 7
13	96± 8	102± 10	99± 9	94± 8
20	119± 11	109± 20	110± 18	108± 7
Lactation day 3	120± 7	125± 9	130± 19	126± 12
8	132± 12	134± 18	142± 20	128± 16
14	142± 19	139± 21	138± 21	132± 30
Energy intake, ave kcal/(100g body wt · day)				
Gestation	32± 2	31± 2	33± 1	31± 3
Lactation	41± 2	39± 3	43± 2	41± 3
Maternal wt. gain, g thru 20 d gestation	154± 16	134± 20	145± 21	135± 17
Litter size (parturition), n	14± 2	12± 3	13± 4	14± 2
Pup wt., g				
Lactation day 1	7.7± 0.5	7.5± 0.4	7.6± 0.4	7.1± 0.3
Lactation day 15	34 ± 4^{a}	31 ± 3^{ab}	34 ± 4^{a}	29 ± 2^{b}

Values are means± SD. Values in the same row with different superscripts are significantly different ($P<0.05$) from each other. If any letter combination matches, the difference between means is not significant. Effects of ethanol, Cu, and ethanol+Cu on the average wt. of 15 day-old pups are $P<0.05$, NS and NS, respectively.

Table 2. Influence of ethanol and copper on maternal and offspring copper content of liver, kidney, spleen and heart

Tissue Cu	Diet, % kcal from ethanol				Significance levels		
	3.75 mg Cu/l		0.75 mg Cu/l				
μg/g	0	30	0	30	Ethanol	Cu	Ethanol+Cu
Dams							
Liver	4.8 ± 0.3^{a}	4.6 ± 0.3^{ab}	4.4 ± 0.3^{b}	3.7 ± 0.3^{c}	<0.005	<0.001	<0.05
Kidney	6.6 ± 1.4^{a}	5.7 ± 0.5^{a}	4.2 ± 0.5^{b}	4.2 ± 0.7^{b}	NS	<0.001	NS
Spleen	8.3 ± 0.6^{a}	9.2 ± 0.7^{a}	6.6 ± 1.0^{b}	6.6 ± 1.0^{b}	NS	<0.001	NS
Heart	23.1± 0.9	23.8± 2.4	22.7± 1.0	23.0± 1.2	NS	NS	NS
Offspring							
Liver	43 ± 16^{a}	27 ± 5^{b}	43 ± 19^{a}	22 ± 4^{b}	<0.005	NS	NS
Kidney	2.7± 0.2	2.6± 0.2	2.6± 0.2	2.5± 0.2	NS	NS	NS
Spleen	10.2± 1.1	8.7± 1.2	10.3± 1.3	9.0± 1.1	NS	NS	NS
Heart	20.9± 0.5	20.4± 0.8	19.8± 1.1	20.0± 0.6	NS	NS	NS

Values are means± SD. Values in the same row with different superscripts are significantly different ($P<0.05$) from each other. If any letter combination matches, the difference between means is not significant. Unit : μg/g dry wt. except liver and kidney(μg/g wet wt.).

per level was not seen with respect to copper concentration in maternal kidney and spleen despite the fact that low dietary copper resulted in a significant depression of copper in these tissues. Copper concentration of maternal heart and pup kindey, spleen and heart were unaffected by either ethanol or diet copper level.

Distribution of copper in pup liver cytosol

The Sephadex G-75 gel filtration elution profile of copper distribution in liver cytosol of 15 day-old pups is shown in Fig. 1. Copper was present in a high molecular weight(greater than approximately 70,000 daltons) protein fraction that eluted at the void volume(fractions 25~33), in an intermediate molecular weight(approximately 30,000 daltons) protein fraction(fractions 34~45) and in a low molecular weight(approximately 10,000 daltons) protein fraction(fractions 46~65). Whanger and Deagen[17)22)] have previously characterized this low molecular weight protein fraction as metallothionein by purification, amino acid analysis, DEAE-Sephacel chromatography and gel electrophoresis. Copper in pup liver cytosol was associated mainly with the metallothionein fraction, similar to others[19)]. The first highest peak in copper concentration of the metallothionein fraction was represented by the control group without ethanol. The second peak was represented by the group fed low copper without ethanol. The third peak was represented by the control copper plus ethanol group. The fourth lowest peak was represented by the low copper plus ethanol group.

Distribution of zinc in pup liver cytosol

As shown in Fig. 2, zinc was present in a high molecular weight protein fraction(fractions 25~37), in an incompletely-seperated fraction of somewhat lower molecular weight protein fraction (fractions 38~45), in a metallothionein fraction (fractions 46~65), and in a very low molecular weight(approximately 1,000 daltons or less) protein fraction(fractions 67~80). Zinc concentration of the metallothionein fraction in pup liver

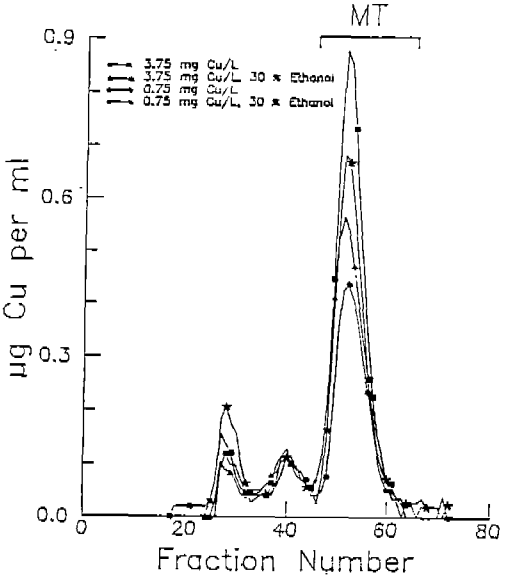

Fig. 1. Distribution of copper in pooled liver cytosol of 15 day-old pups.
MT is the metallothionein fraction.

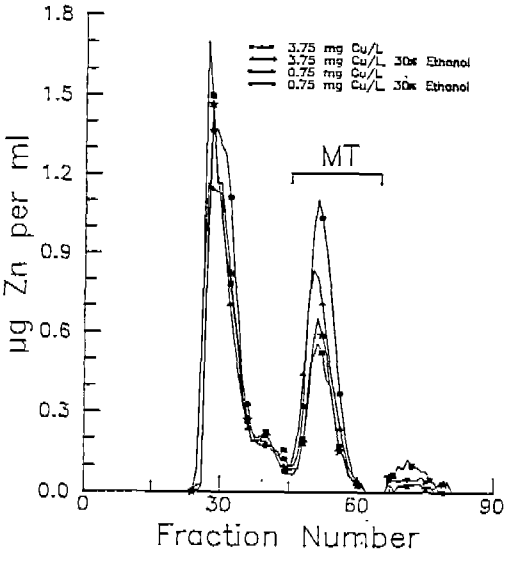

Fig. 2. Distribution of zinc in pooled liver cytosol of 15 day-old pups.
MT is the metallothionein fraction.

cytosol was inversely related to copper concentration of the metallothionein fraction. For example, the first highest peak in zinc concentration of the metallothionein fraction was represented by the low copper plus alcohol group. The second peak was represented by control copper plus alcohol group. The third peak was represented by the group fed low copper without alcohol. The fourth lowest peak was represented by the control group without alcohol.

DISCUSSION

In the present study, the hypothesis was that the known antagonistic effect of alcohol on liver copper concentration[2)3)] could be seen within a period of weeks rather than in months if alcohol ingestion, low dietary copper intake and high metabolic demand for copper, represented by pregnancy and lactation, were simultaneously present. The fact that maternal alcohol ingestion for five weeks depressed maternal liver copper concentration only when dietary copper was low(interactive effect $P < 0.05$) supports this hypothesis. In pups, maternal ethanol ingestion depressed pup liver copper concentration regardless of dietary copper level. The reason why pup liver copper did not mimic the interactive effect seen in maternal liver may be related to the fact that copper utilization is incompletely developed during the neonatal period[23)]. It is also possible that a measurement of total pup liver copper was too insensitive to uncover the interactive effect seen in maternal liver. However, a redefinition of pup liver copper concentration in terms of the protein metallothionein did reveal the interaction between copper and alcohol seen in mature liver. This latter observation illustrates the important role that metallothionein plays in immature rat liver with respect to initial uptake and temporary storage of copper unlike that for mature liver[12)]. These attributes of immature liver metallothionein help to explain why the copper content of liver cytosol, mostly in the form of metallothionein in neonatal rat liver[19)], is depleted most rapidly in a developing copper deficiency compared to all other subcellular liver fractions[24)]. In addition, the low copper plus ethanol group showed the highest peak in zinc concentration of metallothionein fraction.

Overall, our results show that exaggeration of a low copper status into a copper deficiency by adding ethanol to the maternal diet was specific for both maternal and pup liver because copper concentration of kidney, spleen and heart was unaffected by ethanol. This result is probably related to the fact that the liver is the key organ regulating internal copper homeostasis[4)]. At the present time, no one has attempted to define a mechanism to explain why alcohol specifically depresses copper concentration of liver relative to all other tissues. A major reason why this is true is because until now the antagonistic effect of alcohol on liver copper status appeared to be a chronic effect of alcohol. Now that it is shown that this need not be the case, additional studies will be hopefully attempted to define a mechanism for the copper and alcohol interaction seen in pregnancy and lactation. Pursuit of the answer to this question is relevant because the liver regulates copper distribution to all other tissues[25)]. If alcohol, for example, caused the liver to increase its synthesis of the copper transport protein, ceruloplasmin, it could explain why other tissues appear to be unaffected by alcohol in the face of a developing depletion of liver copper. If liver copper concentration reached a critically low level, exacerbated by a preexisting dietary copper deficiency and high metabolic demand for copper, it could lead to a significant impairment of several important metalloenzymes known to require copper[25)]. In the present

study it is shown that ethanol ingestion during pregnancy and lactation is an excellent model to pursue such question.

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임신과 수유기간 동안 Alcohol과 저 Copper 식이가 어미와 새끼 쥐 간의 Copper 수준에 미치는 영향

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국문초록

임신과 수유 기간 동안 alcohol의 섭취가 어미와 새끼 쥐의 간내 copper 유용에 미치는 영향을 연구하기 위하여 ethanol(0 혹은 30%의 kcal)과 copper(0.75 혹은 3.75mg/l 식이) 함량에 차이를 둔 식이조성으로 alcohol과 copper의 상호작용을 규명하는 factorial experiment를 수행하였다. 어미 쥐의 열량 섭취와 체중은 식이들에 의해 영향을 받지 않았다. 식이내 copper양이 적을 경우 alcohol을 섭취하지 않은 실험군과 비교하여 alcohol을 섭취한 실험군인 어미 쥐의 간 copper농도가 더욱 감소됨으로써 alcohol과 copper의 상호작용을 보여주었다. 새끼 쥐에게서는 식이내 copper 수준에 관계 없이 어미 쥐의 alcohol 섭취가 간의 총 copper 농도를 감소시켰으며 어미 쥐 간에서 보여진 alcohol과 copper의 상호작용은 metallothionein의 copper 농도에 반영되었다. Metallothionein의 zinc 함량은 metallothionein의 copper 함량과 반비례적인 관계를 보여주었다. 이상의 결과로 임신과 수유기 동안 식이내 copper 함량이 적을 경우 alcohol 섭취는 어미와 새끼 모두에게 copper 결핍을 초래할 수 있다는 것을 시사해준다.