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Effect of Dietary Folate on Hyperhomocysteinemia and Cellular Toxicity Induced Alcohol Administration in Rat Liver

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Abstract

The critical role of folate in the remethylation pathway for methionine synthesis from homocysteine has been well documented. Hyperhomocysteinemia resulting from inadequate folate nutrition has been implicated in increased incidence of macrovascular diseases, colorectal cancer, neural tube defects, etc. Chronic exposure to ethanol impairs folate nutrition and one-carbon metabolism in the liver, which often results in fatty liver due to a defective remetylation process. This study was carried out to investigate the chronic effects of moderate levels of alcohol and dietary folate on plasma homocysteine levels, and on histopathology and biochemical functions of the liver. Rats were raised on experimental diets with three levels of folate (0, 2, 8 mg/kg diet), and 50% ethanol (1.8 ml/kg body weight) was administered intragastically by intubation tubes three times a week for 10 weeks. Plasma homocysteine concentrations were found to be significantly influenced by dietary folate intake and alcohol administration. Among all treatment groups, plasma homocysteine levels were the highest in the animals receiving a combined treatment of folate deficient diet and alcohol administration. Plasma homocysteine concentrations were negatively correlated with folate concentration in the plasma ($p<0.01$) and liver ($p<0.05$). Among alcohol treated rats, increase in plasma homocysteine values due to macrovascular and microvascular fatty changes and spotted necrosis were observed more frequently in folate-deficient animals diet than those on folate-adequate and folate supplemented diets in alcohol-treated rats. These results indicate that folate supplementation above the recommended level might be beneficial in the prevention of alcohol-related hyperhomocysteinemia and abnormal histologic changes in the liver.

Keywords: liver cellular toxicity, homocysteine, alcohol, dietary folate, liver folate

Folate is the water-soluble vitamin, which required for the process of conversion of homocysteine to methionine[1-5]. If in the case of depletion of folate, remethylation from homocysteine to methionine make a problem, and homocysteine is accumulated in the cells, therefore hyperhomocysteinemia is revealed[1-3,6,7]. Hyperhomocysteinemia is an independent risk factor of macrovascular disease[3,6,8-11] and related to several diseases, such as neural tube defect[4,5,12-16] and cancer[5,12], which are caused by folate depletion.

Alcohol is known as the inhibitor of intake, absorption, storage and secretion of folate[17-24]. Acetaldehyde, alcohol metabolite, accelerates degradation of folate, and folate level in the serum is decreased, so that the concentration of homocysteine in the blood is increased[25,26].

Liver plays a role in the alcohol and folate metabolism[21,22,27-29]. Alcohol abuse makes fatty infiltration in the hepatocyte, followed by fatty liver[29] and enzyme activation[22]. If folate is depleted, methionine synthesis, which is needed in the secretion of triglyceride, is disturbed. It aggravates the fatty liver[12,22,25]. If folate depletion and alcohol abuse are combined, remethylation is augmented, so it makes liver damage easily, but folate repletion decreases the degree of liver damage.

The aim of this study is that we reveal the effects of dietary folate and alcohol administration on plasma homocysteine and hepatic structure and function by using the rats, and that folate repletion will be a benefit to the plasma homocysteine and liver function.

Plasma Homocysteine

In this experiment, we observed that the concentration of plasma homocysteine was the highest in the deficient of diet folate level, and lower in the adequacy and supplement status (Fig. 1).

In the previous studies about plasma homocysteine related to folate status, the depletion of diet folate caused elevation of plasma homocysteine level[31-33].

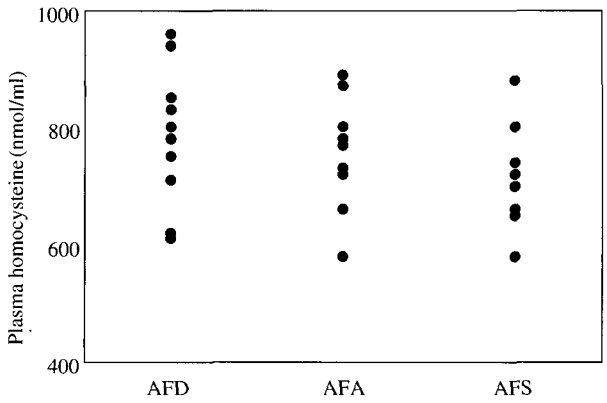

Fig. 1. Distribution of plasma homocysteine concentrations on experimental diets at 10 weeks

Table 1. Plasma homocysteine levels of rats on experimental diets (nmol/ml)

Diet group[1)]	4 wks	7 wks	10 wks
AFD	[2)]676.4±39.0$^{NS3)\alpha 4)}$	713.1±18.6$^{\alpha\beta}$	782.4±28.5$^{\alpha 5)\beta}$
AFA	613.7±41.0$^{\alpha}$	681.0±31.3$^{\alpha\beta}$	758.7±28.2$^{ab\beta}$
AFS	637.0±37.5^{NS}	671.8±31.2	707.9±28.0^{b}
Significant factor		A, B[6)]	

[1)]AFD: Alcohol administered folate deficient group, AFA: Alcohol administered folate adequate group, AFS: Alcohol administered folate supplemented group, [2)]Mean±Standard error, [3)]Not significant at $P<0.05$ by Duncan's multiple range test, [4)]Values with different Greek letters in the same folate levels in diet are significantly different at $p<0.05$ by Duncan's multiple range test, [5)]Values with different alphabets in the same feeding period are significantly different at $P<0.05$ by Duncan's multiple range test, [6)]Statistical significance of factors was analyzed by 3-way ANOVA, A: Effect of folate levels in diet was significant at $P<0.05$, B: Effect of feeding period was significant at $P<0.05$

The higher the concentration of plasma folate is, the lower the concentration of homocysteine[31,34,35]. Folate plays a role of coenzyme in the remethylation process, so if it is depleted, homocysteine does not convert to methionine, and then plasma homocysteine level increases[5,7,13-15].

As the experimental diet period was longer, plasma homocysteine level was increased (Table 1). The folate-deficient and adequate diet increased the concentration of plasma homocysteine significantly. This result agreed with other reports[33,38,39] that the concentration of plasma homocysteine increased with aging. When the folate was supplemented four times to recommendation value, the concentration of plasma homocysteine was increased with breeding period, however 10 week-plasma homocysteine did not increase significantly unlike folate-deficient and folate-adequate diet. We supposed that this result was because of alleviation of increase of homocysteine with aging in the plasma or liver tissue.

There was a report that the risk of acute myocardial infarct was higher 3.4 times in the 12% higher than normal range of homocysteine[8]. This revealed that if homocysteine level was slightly decreased, homocysteine-related diseases would have lower incidence rate.

Table 2. Pearson's correlation analysis of plasma and liver folate concentrations with plasma homocysteine concentrations

	Plasma homocysteine	
	r[1)]	p-value
Plasma folate	−0.3560	0.0002
Liver folate	−0.3304	0.0113

[1)]Pearson's correlation coefficient

Table 3. Plasma GOT and GPT activities of rats at 10 weeks

Diet group[1)]	GOT (U/L)	GPT (U/L)
AFD	[2)]97.96±13.98	19.53±2.58
AFA	77.09±9.24	16.51±1.25
AFS	81.46±7.48	18.46±1.61
Significant factor[3)]	NS	NS

[1)]AFD: Alcohol administered folate deficient group, AFA: Alcohol administered folate adequate group, AFS: Alcohol administered folate supplemented group, [2)]Mean±Standard error, [3)]Statistical significance of factors was analyzed by 2-way ANOVA, NS: Not significant at $P<0.05$

The Relation of Folate Status in the Plasma and Liver with the Concentration of Plasma Homocysteine

As we compared plasma homocysteine with the plasma folate status, in the case of folate-deficient diet plasma folate level was low, and plasma homocysteine level was high, more folate diet contained when higher the plasma folate status was, and the lower plasma homocysteine status was. This result agreed with other reports about the relation with plasma homocysteine and folate[7,40].

Table 2 shows that plasma folate had a negative correlation with plasma homocysteine ($r = -0.35$, $p<0.001$). As the plasma folate level was increased, the plasma homocysteine level was significantly decreased.

In 10 weeks of experimental diet, this study also showed that liver folate level had a significant negative correlation with plasma homocysteine ($r = -0.33$, $p<0.05$). This result was not shown at any other

Table 4. Hepatocellular histological changes in ethanol administered rat at 10 weeks Number of animals (%)

Diet group[1)]	Fatty change macrovesicular		Fatty change microvesicular		Liver cell dysplasia		Spotty necrosis	
	+[2)]	−	+	−	+	−	+	−
AFD (n=12)	10 (83.3)	2 (16.7)	12 (100.0)	0 (0.0)	10 (83.3)	2 (16.7)	10 (83.3)	2 (16.7)
AFA (n=12)	3 (25.0)	9 (75.0)	9 (75.0)	3 (25.0)	8 (66.7)	4 (33.3)	5 (41.7)	7 (58.3)
AFS (n=9)	3 (33.3)	6 (66.7)	6 (66.7)	3 (33.3)	9 (100.0)	0 (0.0)	5 (55.6)	4 (44.4)
	P=0.010[3)]		P=0.109		P=0.444		P=0.106	

[1)]AFD: Alcohol administered folate deficient group, AFA: Alcohol administered folate adequate group, AFS: Alcohol administered folate supplemented group, [2)]Degree of histological change: −, normal; +, abnormal, [3)] Chi-square analysis p value

reports.

In this experiment, plasma folate level of folate-adequate and folate-supplemented groups did not increase after 7 weeks on experimental diet feeding (data not sown). We supposed that this result was because of saturation of plasma folate pool.

Liver Function and Pathologic State

We measured plasma GOT and GPT to examine whether alcohol intake and diet folate status affected biochemical function of the liver. Alcohol intake and diet folate did not deteriorate the liver function such as GOT and GPT (Table 3). In folate-deficient group the mean of GOT and GPT of folate deficient group was higher than other groups, but variation was too large, therefore not significant.

Liver damage, such as fatty change, dysplasia, spotty necrosis, etc., was observed in folate deficient alcohol administered rats at 10 weeks, but severe pathologic change was not noticed (Table 4).

Fatty change was shown most in the folate-deficient group, and frequency of macrovesicular fatty change was higher than other two groups. Mezey[29] reported that in alcohol abuse patients without any symptom of liver disease, 56% of them had fatty infiltration. Alcohol metabolism increases the ratio of NADH/NAD in the hepatocyte. This phenomenon inhibits oxidation of fatty acid and accelerates the synthesis of fatty acid in the liver[29]. In addition, on the edge of depletion of folate the synthesis of methionine for triglyceride secretion is inhibited, so fatty liver is aggravated[22]. Spotty necrosis was prominent in the folate-deficient group. Chronic alcohol abuse accelerates oxidation of liver tissue and induces necrosis[29]. Folate acts as a coenzyme which participates in the synthesis of glutathione that play a role of reducer in vivo. Therefore, in the folate deficiency, the oxidation of liver tissue is able to be promoted[22].

As a result of this study, fatty change and spotty necrosis of liver were observed in the case of folate deficient alcohol administered rats. Halsted[41] reported that liver played a role in the alcohol metabolism and folate metabolism and storage, so excess alcohol uptake and folate deficiency made a synergic effect on liver damages. Matthias[30] knew that alcohol made folate storage status was reduced, and inhibited folate to convert to coenzyme form, then made a trouble on the metabolism of homocysteine. More glutathione was needed for alcohol metabolism, so methionine, which is precursor of glutathione, was consumed exceedingly, and then elevated the concentration of homocysteine[30]. Also liver damage by alcohol disturbed the metabolism of homocysteine and it reduced glutathione synthesis, made liver damage, and caused the vicious circle[22,25]. On this way alcohol and folate deficiency impaired single carbon pathway, and elevated the level of plasma homocysteine.

In the state of folate deficiency, small alcohol intake could make a fatty change and spotty necrosis of liver tissue. However enzyme activation was not noticed.

In this study, we raised rats for 10 weeks with alcohol administration and difference of dietary folate lavel for plasma folate and homocysteine level. Plasma homocysteine level was highest in the folate-deficient group. The elevation of plasma homocysteine was inhibited by folate supplementation. In the histologic examination of liver tissue, alcohol intake and folate deficiency group had more severe fatty liver and spotty necrosis. It was thought that this result was caused due to elevation of plasma homocysteine.

In conclusion, alcohol and diet folate level make a effect on the plasma homocysteine status. As the duration of alcohol is longer, homocysteine status will be worsening. We expect that more studies about

folate and homocysteine should performed in future, and it will reveal the relation of homocysteine and folate followed to find out the cause of homocysteine -related disease.

Method

Experimental Animals and Diet

Twelve of Sprague-Dawley male rats were used. Mean body weight was 146.0±1.1 g. We divided control group into 3 groups by diet folate amounts, such as depletion, adequacy and supplement. We made them all to be alcohol abuse, and bred them for 4, 7 and 10 weeks.

Diet was made by mixing corn, sugar, casein, soy bean, cellulose, mineral compound and vitamins except for folate. Folate status in the diet was 0, 2, and 8 mg each group by Halsted *et al.*[30]. Alcohol, 50% ethanol, was taken 0.18 ml per 100 g body weight by feeding tube three times a week.

Sample Preparation

All rats were starved for 12 hours, exposed to ether, and sampled by heart puncture with syringe dealt with 3.8% sodium citrate before rat samples used. The blood was centrifuged for 30 minutes in the environment of 4°C, 800 rpm. The plasma for liver function test was immediately analyzed after sampling it. Another plasma for folate and homocysteine was kept in a freezer at −70°C. After sampling the blood, we extracted the liver and fixed it with the formalin solution for the histologic examination.

Folate Analysis

Plasma folate was measured by microorganism analysis using Lactobacillus casei (ATCC 7469)[28]. All samples were mixed with Folic Acid Casei Medium (Difco Company). All tubes were inoculated with Lactobacillus casei, and kept in 37°C, and then we read the absorption level in 600 nm length. We adapted the value into folate standard solution curve and quantified the folate amount.

Plasma Homocysteine Analysis

10 μl of plasma was collected in the reaction vial, and evacuated and dried with Pico. Tag work-station (Waters, Japan). Performic acid 10 μl, which was made by formic acid and hydrogen peroxide 19 to 1 ratio, was added, and left in room air for hyper oxidation. It was redried and evacuated, followed by adding 0.5% phenol-HCL 200 μl in the reaction vial and hydrolyzing for 24 hours at 110°C with nitrogen plugging. After vacuum dry, redry solution (methanol : sodium acetate:triethylamine = 2 : 2 : 1) was added. It was derived for one hour, and was centrifuged with 0.1% TEA and 1.4 mM sodium acetate containing 6% acetonitrile (pH 6.4). Supernatant was collected and filtered by 0.45 μm filter (HV type). HPLC was used to analysis of homocysteine concentration (column : Pico · Taq 8.5 mm × 300 mm).

Liver Function Test and Hitologic Examination

We evaluated the liver function test by measuring GOT and GPT with GOT, GPT kit (Young Dong Pharmacy) using Reitman-Frankel method.

Liver tissue fixed with formalin solution was embedded in paraffin and cut with microtome by 4 μm width. It was stained by Hematoxylin-eosin and PAS method. We examined the frequency of fatty change, dysplasia and spotty necrosis with light microscope.

Data Analysis

All data were presented as mean and standard error. SPSS (ver 11.0) was used for statistical analysis. To evaluate the significant factors of dietary folate level and breeding duration, two-way ANOVA was used, and one-way ANOVA was used for GOT, GPT value. After statistic analysis, significant data were verified by Duncan's multiple range test, whether the mean value was significantly different. The relation of plasma folate and homocysteine was analyzed by using Pearson correlation analysis. Liver histologic examination was analyzed by using χ^2-analysis.

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