Trans-HHS Workshop:
Diet, DNA Methylation Processes and Health

Metabolic Interactions of Alcohol and Folate[1,2]

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ABSTRACT The goals and objectives of these studies, conducted over the past 30 y, were to determine: *a*) how chronic alcoholism leads to folate deficiency and *b*) how folate deficiency contributes to the pathogenesis of alcoholic liver disease (ALD). The intestinal absorption of folic acid was decreased in binge drinking alcoholics and, prospectively, in volunteers fed alcohol with low folate diets. Monkeys fed alcohol for 2 y developed decreased hepatic folate stores, folic acid malabsorption and decreased hepatic uptake but increased urinary excretion of labeled folic acid. Micropigs fed alcohol for 1 y developed features of ALD in association with decreased translation and activity of intestinal reduced folate carrier. Another study in ethanol-fed micropigs demonstrated abnormal hepatic methionine and DNA nucleotide imbalance and increased hepatocellular apoptosis. When alcohol feeding was combined with folate deficiency, micropigs developed typical histological features of ALD in 14 wk, together with elevated plasma homocysteine levels, reduced liver *S*-adenosylmethionine and glutathione and increased markers for DNA and lipid oxidation. In summary, chronic alcohol exposure impairs folate absorption by inhibiting expression of the reduced folate carrier and decreasing the hepatic uptake and renal conservation of circulating folate. At the same time, folate deficiency accelerates alcohol-induced changes in hepatic methionine metabolism while promoting enhanced oxidative liver injury and the histopathology of ALD. J. Nutr. 132: 2367S–2372S, 2002.

KEY WORDS: • *folate* • *alcohol* • *methionine* • *alcoholic liver disease*

Alcohol is both a food that provides 8 kcal/g and an intoxicating drug that is consumed in varied amounts by at least two-thirds of Americans. Chronic alcohol addiction affects at least 5% of the U.S. population. These individuals consume alcohol in excess and are at risk for alcoholic liver disease (ALD)[4] (1). Concepts on the role of nutrition in the pathogenesis of ALD have come full circle, from the preponderant belief in alcohol as a sole pathogenic toxin to the understanding that alcohol is a significant component of the American diet, which, when consumed in excess, has profound effects on the availability and hepatic metabolism of many nutrients (2). In this context, it has been recognized for >30 y that folate deficiency is a common clinical sign of chronic alcohol abuse. For example, low serum folate levels were found in as many as 56 (80%) of 70 derelict Boston alcoholics (3) and, together with megaloblastic anemia, in 33 (50%) of a group of 65 Seattle patients with ALD (4). A British study of 84 alcoholics, most with ALD, found that one-third had megaloblastic anemia in association with low red cell and liver folate levels (5). The significant incidences of folate deficiency found in these historical studies of chronic alcoholism suggest a role for folate deficiency in the development of certain complications of alcoholism, in particular ALD. This review will focus on two major areas of interest to our laboratory: the causes of folate deficiency in alcoholism

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[4] Abbreviations used: 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; ALD, alcoholic liver disease; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartyl aminotransferase; BHMT, betaine homocysteine methyltransferase; CDP-choline, cytidine diphosphocholine; choline-P, phosphorylcholine; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate; dUTP, deoxyuridine triphosphate; FBP, folate-binding protein; FD, folate deficient; FDE, FD with ethanol; FS, folate sufficient; FSE, FS with ethanol; GCPII, glutamate carboxypeptidase; GSH, glutathione; GSSG, oxidized GSH; MAT, methionine adenosyltransferase; MDA, malondialdehyde; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; oxo^{8}dG, 8-oxo-2′-deoxyguanosine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, PE methyltransferase; RFC, reduced folate carrier; SAH, *S*-adenosylhomocysteine; SAHH, SAH hydrolase; SAM, *S*-adenosylmethionine; SM, sphingomyelin; TNF-α, tumor necrosis factor-α.

and the potential role of folate deficiency in the pathogenesis of ALD.

DISCUSSION

Causes of folate deficiency in alcoholism

While dietary folate inadequacy is common in derelict alcoholics (3), clinical studies and experiments with animal models demonstrate significant effects of chronic alcohol exposure on folate homeostasis in the body. Briefly, folate homeostasis involves a two-stage process of initial hydrolysis of polyglutamyl dietary folates followed by transport of the monoglutamyl derivative across the epithelial cells of the upper small intestine, uptake and metabolism in the liver, an enterohepatic circulation and excretory regulation by the renal tubule. Folate-specific proteins regulate each of these processes. Thus, the intestinal absorption of dietary polyglutamyl folates requires a brush border hydrolase, now known as glutamate carboxypeptidase (GCPII) (6), and the reduced folate carrier (RFC) (7) for transport across the brush border and basolateral membranes. Both folate-binding protein (FBP) and RFC are involved in the binding and transport of monoglutamyl folate, principally 5-methyltetrahydrofolate (5-MTHF), across the liver plasma membrane and kidney tubule brush border membranes (8,9). Our laboratory has been involved with others in the study of these processes, both in health and under the influence of chronic alcohol exposure.

Small intestine. In initial clinical studies from our laboratory that used the triple lumen tube perfusion method, we found that the mean jejunal uptake of [^{3}H]folic acid was reduced in recently drinking and malnourished chronic alcoholics to <20% compared to 35% after 2 wk of alcohol withdrawal and hospital diet (10). In a subsequent prospective study, we showed a significant reduction in the jejunal uptake of [^{3}H]folic acid in alcoholic volunteers fed a combination of low-folate diet and alcohol for 6 wk (11). In subsequent experiments in a macaque monkey model, we induced folate deficiency with low liver folate levels after 2 y of feeding alcohol at 50% of kcal with a normal folate diet. We showed intestinal malabsorption in these monkeys by increased 5-d fecal excretion and decreased urine excretion and decreased calculated total body retention of radioactive label after intragastric administration of [^{3}H]folic acid compared to the nonalcoholic control group (12). These findings in alcohol-fed monkeys have been confirmed by others (13).

Liver uptake and renal excretion. We studied these aspects of folate homeostasis in the same macaque monkeys. After 2 y of feeding, a trace amount of [^{3}H]folic acid was injected into each animal, and liver biopsies were obtained from each pair sequentially over 4 d. Among the alcohol-fed monkeys, we found decreased hepatic folate and tritium concentrations and increased urine tritium excretion. However, the relative amounts of reduced, methylated, formylated and polyglutamylated folates were similar in liver homogenates from each group. These studies suggested that the hepatic uptake and/or retention of folate is reduced in alcoholic livers, whereas intracellular folate metabolism is unaffected (14). After 4 y of feeding, the same monkeys received another tracer dose of [^{3}H]folic acid, and radioactivity was quantified in daily collections of urine and stool for the next 25 d. Both urine and fecal tritium excretion were increased in alcohol-fed animals during the first 3 d, whereas longer-term rates of tritium excretion were unchanged and terminal liver tritium and folate levels were significantly reduced. While confirming decreased hepatic uptake and/or retention of folic acid in the alcohol-fed monkeys, these studies also suggested that relative folate deficiency was caused in part by reduced renal tubular reabsorption (15). Others demonstrated increased urinary folate excretion in chronic alcoholic patients and alcohol-fed rats (16–18).

Other recent work from our laboratory has been directed at the role of regulatory proteins in folate homeostasis and the effects of chronic alcohol exposure on these proteins. We defined the biochemical and molecular characteristics of intestinal GCPII, which are identical in pig and human (6,19–21). The activity of GCPII was decreased in jejunal brush borders of chronic alcohol-fed pigs together with a decrease in hydrolysis of polyglutamyl folates perfused in the jejunum (22,23). We demonstrated the absence of FBP transcripts and activity in pig jejunum and its presence in liver and kidney (8,24). In studies of micropigs fed alcohol at 40% of kcal for 12 mo, we found RFC activity in ascending amounts in intestine, kidney and liver. Compared to control pigs, alcohol-fed animals displayed decreased transcripts and activity of RFC in the jejunum but not in liver or kidney (9).

In summary, these studies in man, monkey and pig indicate that chronic alcohol ingestion reduces the intestinal absorption of both dietary polyglutamylated folate and its monoglutamyl folic acid derivative together with reduced expressions of both GCPII and RFC. Our data from the monkey model also show disruption of hepatic and renal homeostasis. All of these factors may contribute to folate deficiency during chronic exposure to excessive amounts of alcohol.

Pathogenesis of ALD and altered methionine metabolism

Understanding of the pathogenesis of ALD has been aided by the use of animal models in which alcohol is provided in the diet or via gastric intubation as a varied percentage of kcal in eucaloric substitution for carbohydrate while retaining all other essential nutrients (25,26). Our laboratory developed the alcoholic micropig to study the pathogenesis of ALD. In contrast to other species, the pig consumes alcohol voluntarily and completely in the diet. In our initial studies, castrated male Yucatan micropigs were fed 40% of kcal as alcohol or cornstarch control, polyunsaturated corn oil and all other essential nutrients. Alcohol-fed micropigs developed progressive histopathology including steatonecrosis and inflammation at 5 mo, interstitial fibrosis by 12 mo and early cirrhosis by 21 mo, together with the accumulation of intrahepatic protein adducts of the alcohol metabolite acetaldehyde and lipid oxidant products of malondialdehyde (MDA) (27,28). These and many other detailed studies have led to the concept that ALD represents a process of oxidant liver injury that is associated with alcohol metabolism, acetaldehyde accumulation and enhanced collagen synthesis, all of which are mediated by tumor necrosis factor-α (TNF-α) and other cytokines (29–31).

Other studies have established the concurrence of abnormal intrahepatic methionine and choline metabolism with ALD. As illustrated in **Figure 1**, cellular methionine metabolism is regulated in part by the availability of 5-MTHF, which is substrate with cofactor vitamin B-12 for the methionine synthase (MS) (5-methyltetrahydrofolate-homocysteine *S*-methyltransferase; EC 2.1.1.13) reaction that generates methionine from homocysteine. In the alternate salvage pathway for methionine, choline is precursor to betaine, which is the substrate for betaine homocysteine *S*-methyltransferase (BHMT; *S*-EC 2.1.1.5). Methionine is converted to *S*-adenosylmethionine (SAM) by methionine adenosyltransferase

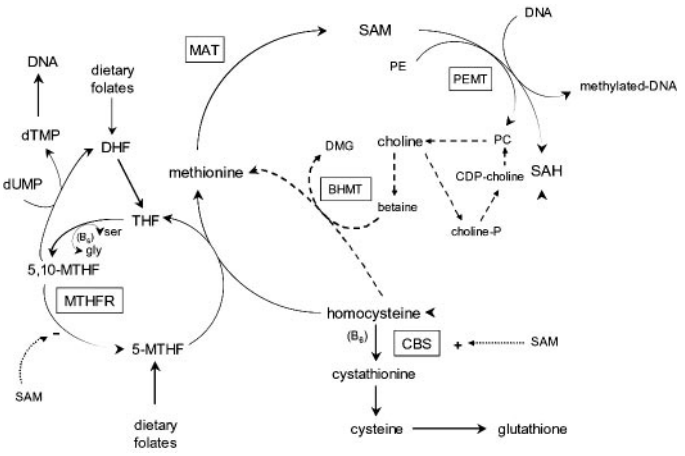

FIGURE 1 Folate and methionine metabolism in the liver. 5-Methyltetrahydrofolate (5-MTHF) is substrate for the methionine synthase (MS) reaction that generates methionine from homocysteine. In an alternate salvage pathway, betaine, a product of choline metabolism, is the substrate for betaine homocysteine methyltransferase (BHMT). The precursors of choline include phosphatidylcholine (PC), cytidine diphosphocholine (CDP-choline) and phosphorylcholine (choline-P). Methionine is converted to *S*-adenosylmethionene (SAM) by methionine adenosyltransferase (MAT). Through reactions that include DNA methylation and the synthesis of PC from phosphatidylethanolamine (PE) by PE methyltransferase (PEMT), SAM is converted to *S*-adenosylhomocysteine (SAH), which is also upregulated by synthesis from homocysteine through the reversible SAH hydrolase reaction. SAM regulates the synthesis of glutathionine (GSH) by upregulation of cystathionine β synthase (CβS) and the homocysteine transsulfuration pathway. SAM provides negative regulatory feedback to the MTHFR reaction that converts 5,10-MTHF to 5-MTHF. DHF, dihydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; gly, glycine; ser, serine; THF, tetrahydrofolate; TS, thymidylate synthase.

(MAT; EC 2.5.1.6), which is expressed in liver as MAT1A and in other tissues as MAT2A. Through many methyltransferase reactions, including DNA methylation and the synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) by PE methyltransferase (PEMT; EC 2.1.1.17), SAM is converted to *S*-adenosylhomocysteine (SAH), which also may be increased by synthesis from homocysteine through the reversible SAH hydrolase (SAHH) reaction.. Elevation in homocysteine, decreased SAM synthesis and increased SAH therefore reduce the SAM:SAH ratio, which is a convenient expression of the opposing effects of each metabolite (32). For example, recent studies show that SAH exerts opposing or inhibiting effects on SAM-dependent DNA methylation (33). In liver, kidney and small intestine, SAM regulates the synthesis of the principal antioxidant glutathione (GSH) by its upregulation of the homocysteine transsulfuration pathway (34). GSH is oxidized to GSSG, and the GSH:GSSG ratio may be considered an accurate measure of overall oxidative state (35). SAM provides negative regulatory feedback to the methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) reaction that converts 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-MTHF. Thus, adequate SAM ensures sufficient 5,10-MTHF for the thymidylate synthase (EC 2.1.1.45) reaction, which ensures nucleotide balance, specifically of deoxyuridine monophosphate (dUMP) and deoxythymidine monophosphate (dTMP).

The metabolism of choline is integrally related to methionine metabolism and the integrity of membrane phospholipids. Because choline is a principal source of betaine substrate for BHMT, endogenous choline deficiency can be induced by increasing the activity of this reaction. For example, dietary induction of folate deficiency in the rat resulted in significant reduction in hepatic choline and its metabolite phosphorylcholine (choline-P) (36). Choline-P also is a precursor for cytidine diphosphocholine (CDP-choline), which reacts with intracellular ceramide for maintaining membrane sphingomyelin (SM) (37). In consideration of these metabolic relationships, it could be predicted that significant folate deficiency would limit the MS reaction, resulting in decreased hepatic methionine and SAM and hence decreased DNA methylation and endogenous production of PC. At the same time, enhancement of the compensatory BHMT pathway would perturb choline metabolism, potentially reducing choline and its precursors, choline-P, CDP-choline and PC, thereby affecting membrane lipid composition, including the endogenous synthesis of SM.

Several studies demonstrated the interactions of abnormal methionine metabolism with ALD. Three different groups described reduced MS activity in alcohol-fed rats, in which the products methionine and SAM were either reduced or, alternatively, preserved by enhancement of the BHMT salvage pathway (38–40). Prolonged alcohol feeding in the baboon decreased hepatic levels of SAM and GSH, an effect that was attenuated by coadministration of exogenous SAM (41). Other studies in the same baboon model showed reduced SAM-dependent PEMT activity and PC levels, while the severity of alcoholic liver injury was attenuated by PC in the form of soy lecithin (42,43). A recent study using the intragastric alcohol-fed rat model demonstrated decreased hepatic SAM and SAM:SAH ratio, together with decreased MAT1A activity and increased DNA strand breaks (44). A recent study of liver biopsies of patients with ALD demonstrated decreased mRNA levels of MAT1A, MS and BHMT (45). A multicenter European trial demonstrated the efficacy of SAM in improving survival in patients with ALD (46).

Relationship of abnormal hepatic methionine metabolism to ALD in the micropig

We conducted two separate studies on the effect of chronic alcohol feeding on hepatic methionine metabolism in the micropig, one previously published (47) and the other summarized here. In the published study, juvenile uncastrated males were allocated to two groups of six each to receive control diet or alcohol diet at 40% of kcal for 12 mo that included adequate levels of all nutrients, including methionine and choline, and excess folate at 11.8 μg/kg body weight per day (47). In the alcohol group, a mean intoxicating blood level of 171.6 mg/dL was achieved 2 h after the last daily feeding. In contrast to our original study in which features of ALD were induced by 12 mo of alcohol diet in castrated male micropigs (27), there were no changes in serum aminotransferase levels, and terminal liver histology was similar in the control and alcohol-fed groups. The protective effect of testosterone on processes of liver injury was substantiated by further analysis of levels of sex steroids in the two different studies of castrated and uncastrated males (47,48). Thus, results from the study that used intact uncastrated male micropigs represent the effect of 12 mo of feeding of excessive alcohol on methionine metabolism in the absence of histologic features of alcoholic liver injury (47).

Taken at monthly intervals, serum homocysteine levels increased threefold in the alcohol group by 2 mo and remained

at this level throughout the experiment, with a corresponding decrease in serum methionine levels. Although excessive dietary folate intake resulted in similar terminal liver folate levels, there were clear and significant differences in methionine metabolism between control and alcohol-fed animals. Comparing the alcohol-fed group to the control group, MS activity was 20% lower, SAM levels were unchanged, SAH was increased by 41% and the SAM:SAH ratio was decreased by 36%. Of interest, terminal serum levels of cystathionine and serine were each deceased in the alcohol-fed animals, consistent with reduction in the transsulfuration pathway of homocysteine degradation. The same liver samples were used to assess hepatocellular nucleotide balance and cell turnover. While deoxyuridine triphosphate (dUTP) levels were unchanged, the dUTP:deoxythymidine triphosphate (dTTP) ratio was increased by 130% in the alcohol-fed group. A linkage between MS activity and the dUTP:dTTP ratio was shown by a significant correlation between the two variables. Because nucleotide imbalance affects DNA integrity and cell turnover, hepatocyte apoptosis and proliferation were each measured in terminal liver biopsies by specific immunohistochemical techniques. The incidence of apoptosis was increased by threefold, while the numbers of proliferating cells in S phase were nonsignificantly increased by sixfold in alcohol-fed micropigs. In summary, this study showed perturbations of methionine metabolism in micropigs fed alcohol with an excess of dietary folate and in absence of folate deficiency or histopathology. Unchanged SAM levels in the face of alcohol-induced reduction in MS suggested enhancement of the compensatory BHMT pathway, whereas increasing SAH was consistent with increased serum homocysteine levels. The linkage of decreased MS activity to nucleotide imbalance could be explained by an increase in MTHFR activity due to loss of SAM regulation. This effect could further reduce the availability of 5,10-MTHF as substrate for dTTP synthesis. The association of nucleotide imbalance with DNA strand breaks, apoptosis and a compensatory increased cellular proliferative response has been shown by others in rats fed low-folate and low-methyl diets (49).

Our more recent micropig study tests the hypothesis that the development of ALD is accelerated by the combination of chronic alcohol exposure and folate deficiency, because each factor plays a significant role in perturbing methionine metabolism. Four groups of intact uncastrated male micropigs at 6 mo of age (Sinclair, Columbia, MO) were fed diets at 90 kcal/kg body weight per day containing polyunsaturated corn oil at 33% of kcal, protein at 2 g/kg body weight per day and cornstarch as carbohydrate or the same diet substituting alcohol for cornstarch at 40% of kcal or 5 g/kg body weight per day. Four different feeding protocols included diets that were folate sufficient (FS; or control) containing excess folate at 14.5 μg/kg body weight, folate deficient (FD) containing no folate, FS with ethanol (FSE), and FD with ethanol (FDE) over a period of 14 wk. Each diet contained sufficient choline at 60.3 mg/kg body weight and methionine at 675 mg/kg body weight and all essential micronutrients in accord with requirements of growing swine (50). The protocol for this study was approved by the University of California Davis Animal Welfare Committee according to the Guide for the Care and Use of Laboratory Animals. Micropigs were group-paired weekly to ingest the same amount as ingested by the FDE group. Blood samples were obtained at 2-wk intervals for measurements of homocysteine values. Animals were killed after 14 wk by administration of isofluorane anesthesia and exsanguination by venipuncture. Terminal plasma samples were used to measure two liver injury enzymes, aspartyl aminotransferase (AST; EC 2.6.1.1), alanine aminotransferase (ALT; EC 2.6.1.2) and MDA as an index of lipid peroxidation. Terminal urine was obtained by bladder puncture and analyzed for 8-oxo-2′-deoxyguanosine (oxo^{8}dG) as an index of DNA oxidation. Terminal liver samples were analyzed for folate, methionine and choline metabolites, activities of MS and BHMT and histology. The data were analyzed by repeated measures of two-way analysis of variance (ANOVA), where the independent variables were folate status (sufficient or deficient), ethanol treatment and time. When interactions were significant, separate subgroup analyses were performed; otherwise, analyses were performed with both variable groups pooled. Correlations were determined by linear regression, and significance was determined by ANOVA.

All of the following findings were statistically significant. Figures in parentheses represent the percentage of change compared to mean values in the control group. During the 14-wk experiment, growth was attenuated in the FSE and FDE groups as compared to FS and FD. Taken at 2-wk intervals, plasma homocysteine levels rose in all experimental groups and at 14 wk were 2.5-fold greater in FDE than in FS control. Terminal hepatic folate levels were reduced by folate deficiency in FD (−50%) and FDE (−45%). While MS activity was reduced by alcohol in both FSE (−35%) and FDE (−26%), BHMT activity was increased by folate deficiency in FD (36%) and FDE (13%). Diet had no effect on hepatic choline levels, while betaine was reduced in FD (−44%) and FDE (−42%), and choline-P was reduced in FSE (−86%) and FDE (−86%). These data suggest that betaine was reduced through activation of the compensatory BHMT pathway, whereas choline was sustained through reduction in its choline-P precursor.

Hepatic SAM was reduced by ethanol in FSE (−28%) and FDE (−32%), while SAH was increased in FDE (113%) and the SAM:SAH ratio was reduced in FDE (−70%), each by the combination of ethanol and folate deficiency. Both GSH (−26 and −35%) and the GSH:GSSG ratio (−37% and −56%) were reduced by ethanol in the FSE and FDE groups. DNA oxidation, expressed as urinary oxo^{8}dG, was increased in FDE (50%) by the combination of ethanol and folate deficiency, while membrane lipid peroxidation, expressed as plasma MDA, was increased by ethanol in FSE (24%) and FDE (19%). The liver injury enzyme AST was increased by ethanol in FSE (57%) and significantly more by the combination of ethanol and folate deficiency in FDE (733%). ALT was increased by ethanol in FSE (110%) and in FDE (180%). Histological evaluation of terminal livers after 14 wk showed no changes in the FS, FD, and FSE groups. However, the livers from the micropigs fed the FDE diet demonstrated significant liver injury with focal steatonecrosis and inflammation in every lobule.

Linear regression ANOVA using all data points for each variable was used to evaluate relationships among different methionine and choline pathway enzymes and their metabolites. An inverse correlation between MS and BHMT activities was consistent with the compensatory increase in BHMT in response to ethanol inhibition of MS. While SAM followed the levels of its precursor methionine, GSH correlated with levels of SAM in keeping with SAM regulation of the transsulfuration pathway. Inverse correlations of GSH with oxo^{8}dG and MDA were each consistent with the major antioxidant role of GSH. A correlation of SAM/SAH with ALT demonstrated the linkage of abnormal methionine metabolism to oxidative liver injury.

We conclude that perturbations of the methionine met-

abolic cycle are clearly associated with oxidative alcoholic liver injury, steatosis and inflammation. Because the present histological effects after 14 wk were magnified by folate deficiency in the presence of alcohol feeding (FDE) but were absent in both groups fed folate-sufficient diets (FS and FSE), the findings suggest that folate deficiency accelerates and folate sufficiency protects against the early development of ALD.

In summary, studies from our laboratory using patients and experimental primates demonstrated the association of chronic alcoholism with the intestinal malabsorption, decreased hepatic uptake and increased urinary excretion of folic acid, contributing over the long term to folate deficiency according to low hepatic folate levels. The pig model demonstrated that chronic alcohol exposure leads to decreased activity of two proteins that regulate folate absorption, intestinal GCPII and RFC. Because clinical chronic alcoholism usually is associated with folate deficiency and hepatic methionine metabolism is influenced by both folate deficiency and alcohol exposure, we explored the possibility that the single and/or combined effects of folate deficiency and chronic alcohol exposure affect the development of ALD. An initial study demonstrated that 12 mo of alcohol exposure with surplus dietary folate resulted in abnormalities in methionine metabolism that were correlated with DNA nucleotide imbalance and increased hepatocellular apoptosis. A subsequent study shows that the combination of FD diet and chronic alcohol exposure accentuates perturbations of hepatic methionine metabolism and accelerates the development of the histologic features of alcoholic liver injury. Additional studies are required to define the mechanisms for the single and combined effects of folate deficiency and chronic alcohol exposure on the pathogenesis of ALD.

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