Abstract

Methyl-donor Enrichment Attenuates Alcohol-induced Liver Injury: Mechanisms of Protective Action

(Under the direction of Ivan Rusyn, M.D., Ph.D.)

Alcohol abuse is a major social as well as clinical problem with nearly 20% of alcoholics developing fibrosis and subsequent cirrhosis of the liver, neither of which have an acceptable cure outside of organ transplantation. Chronic alcohol abuse leads to abnormal hepatic methionine metabolism resulting in decreased levels of S-adenosylmethionine (SAM) which increasing evidence suggests is a critical factor in the development of alcohol-induced liver injury (ALI). The mitigation of disease severity in animal models of ALI has been achieved through dietary supplementation of methionine metabolites including SAM and betaine; however, the mechanism of protection remains unclear. To determine whether methyl-donor supplementation acts through modulating alcohol metabolism, mice were administered a high fat diet (HFD), or methyl-donor (MD) enriched HFD (MDHFD) with or without ethanol for 4 wks using intra-gastric intubation. Attenuation of ALI was achieved in mice administered a MDHFD as compared to those only on a HFD, an effect which was accompanied by an improved GSH:GSSG ratio, reduced hepatic cysteinylglycine levels, reduced liver:body weight and reduced cell proliferation. Blood and liver alcohol concentrations were not different between ethanol-treated groups at sacrifice; however, mean daily urine alcohol concentrations (UAC) were reduced in half by MDHFD. Stomach catalase activity was not different between alcohol
treated groups in the sub-chronic study, suggesting that the lowered UAC by MDHFD was not due to altered first pass metabolism of alcohol in the stomach. Next, we investigated whether MDHFD altered hepatic ethanol metabolism. Alcohol dehydrogenase activity was similar between ethanol treated groups; however, cytochrome P4502E1 activity was reduced in half in mice fed MDHFD containing ethanol. Interestingly, acyl CoA oxidase activity along with catalase activity were elevated in mice administered a MDHFD containing ethanol. In summary, a MD-enriched diet stimulated hepatic ethanol metabolism via catalase resulting in lowered UAC in addition to improved liver detoxification capacity. Since methyl-donor supplements are being considered as potential therapies for ALI, understanding the protective mechanism of action has the potential to increase therapeutic efficacy.
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List of Abbreviations

ADH – Alcohol dehydrogenase
ALD – Alcoholic liver disease
ALI – Alcohol-induced liver injury
ALT – Alanine transaminase
AST – Aspartate aminotransferase
BAC – Blood alcohol concentration
CYP2E1 – Cytochrome P450 2E1
GSH – Reduced glutathione
GSSG – Oxidized glutathione
HCC – Hepatocellular carcinoma (HCC)
HFD – High fat diet
IARC – International Agency for Research on Cancer
LAC – Liver alcohol concentration
MDHFD – Methyl donor enriched high fat diet
NAD – Nicotinamide adenine dinucleotide
NADH – Nicotinamide adenine dinucleotide phosphate
ROS – Reactive oxygen species
SAH – S-adenosylhomocysteine
SAM – S-adenosylmethionine
UAC – Urine alcohol concentration
WHO – World Health Organization
CHAPTER 1
Literature Review

Alcohol abuse and global patterns

Chronic alcohol consumption is a major health issue worldwide and may lead to addiction and damage of almost every organ in the body. Approximately two billion people worldwide consume alcoholic beverages and 76.3 million have diagnosable alcohol use disorders (1, 2). The World Health Organization (WHO) ranks alcohol third among preventable risk factors for premature death in developed nations (3). In the U.S. in 2001, 76,000 deaths were attributable to alcohol; 35,000 of these deaths were due to chronic conditions especially alcoholic liver disease and liver cirrhosis, the remaining deaths were attributable to alcohol related accidents (4). Alcohol consumption is rising in many countries as a result of both increasing numbers of alcohol drinkers and intake of alcohol, especially for women and in regions of rapid economic growth, like east Asia (5). Alcohol is probably the main factor responsible for increased risk of oral cavity and neck cancer recorded in various countries, particularly in central and eastern Europe (6).

The likelihood of developing liver disease in an individual is related to the amount of alcohol consumed daily and duration of consumption over a lifetime. Type of alcoholic beverage (i.e. wine, beer, spirits) consumed has not been correlated with the development of alcohol-related disease (7). Examining drinking patterns is important for understanding how alcohol is intertwined into our society. Alcohol abuse (harmful/hazardous use) is defined as a recurring pattern of high-risk drinking that results in adverse outcomes including personal problems (memory loss, relationship problems, organ damage), problems with others (violence and crime), and problems in society.
(underage drinking, health care costs, economic productivity). Alcoholism, also referred to as alcohol dependence, is defined by a persistent and progressive pattern of abnormally intense alcohol-seeking behavior that results in loss of control over drinking, a preoccupation with drinking, unable to stop drinking, and the development of tolerance or dependence.

Drinking in a manner that will cause intoxication clearly poses risks to the drinker, commonly known as binge drinking. This is a pattern of drinking that brings the blood alcohol concentration (BAC) to 0.08 gram percent within a certain timeframe. For typical adults this pattern corresponds to consuming 5 or more drinks for a male or 4 or more drinks for a female in 2 hours (a “drink” refers to a serving of 12 g absolute alcohol; e.g. one 12-oz beer, one 5-oz glass of wine, or one 1.5-oz shot of distilled spirit). Moderate drinking is defined by consuming up to two drinks per day for men and one drink per day for women or senior adults. While moderate drinking is considered to offer health benefits for some, drinking at this level poses real risks for others with specific susceptibilities (8).

According to the World Health Organization’s (WHO) global burden of disease project, alcohol caused 185,000 deaths of men in developed countries in 2000, whereas moderate alcohol consumption prevented 71,000 deaths in men for the same year. For women in developed countries, 277,000 deaths were prevented compared with the 142,000 caused by alcohol. However, in developing countries, a lower burden of cardiovascular disease and a greater incidence of injuries compared with developed countries led to 1,524,000 deaths in men and 301,000 in women in 2000. Therefore, the global burden of alcohol amounts to 1,804,000 deaths a year, or 3.2% of all deaths a year (9).
Cirrhosis and hepatocellular carcinoma (HCC) prevalence rates are quite useful for interpreting the global burden of chronic disease due to alcohol; however, it is very difficult to separate the etiology of these diseases. Cirrhosis mortality has registered large changes over the last few decades. In the early 1980s, the highest rates of cirrhosis were in Mexico, Chile (around 55/100,000 men and over 14/100,000 women), France, Italy, Portugal, Austria, Hungary and Romania (around 30-35/100,000 men and 10-15/100,000 women) (10). Mortality from cirrhosis has been steadily declining in most countries worldwide since the mid or late 1970’s. In southern Europe, rates in the early 2000’s were decreased by nearly 50% compared to earlier decades. On the other hand, rates have been rising in Eastern European countries since the mid 1990s. Overall, mortality from cirrhosis has shown favorable trends in most countries of the world, as a result of the reduction in alcohol consumption and decreased hepatitis B and C virus infection. The steady upward trends observed in the United Kingdom and central and eastern European countries are attributed to the persistent increase in the prevalence of alcohol consumption (10).

Ninety percent of HCC develop in the context of chronic liver diseases and mainly in patients with cirrhosis (11). Moreover, HCC has been reported as the third most common cause of cancer-related death in the world. HCC is a unique cancer in that it occurs mostly in patients with a known risk factor. Viral hepatitis is the most common cause of HCC worldwide, followed by alcoholic liver disease (ALD) and other causes such as non-alcoholic fatty liver disease, genetic hemochromatosis, and biliary cirrhosis. Eighty percent of HCC cases occur in developing countries, in the Far East and South Asia, where the prevalence of viral hepatitis is higher and economic constraints limit prevention campaigns (11). HCC is one of the few types of cancer that is actually
increasing in frequency and mortality in the U.S.\(^\text{(11)}\). Epidemiological studies of risk factors associated with HCC suggest that heavy alcohol consumption contributes to most HCC cases\(^\text{(12)}\).

Ethanol, which is the type of alcohol found in wine, beer and spirits, is classified by the International Agency for Research on Cancer (IARC) as a “known human carcinogen” when consumed orally\(^\text{(13)}\). Many prospective and case-control studies show a 2-3-fold increased risk for cancer of the oral cavity, pharynx, larynx, and esophagus in people who consume 50 g of alcohol a day (approximately a half bottle of wine, compared to non-drinkers), an effect which is dose dependent \(^\text{(14)}\). The major non-cancer diseases caused by alcohol include alcoholic polyneuropathy, alcoholic cardiomyopathy, alcoholic gastritis, depression and other mental disorders, hypertension, stroke, liver cirrhosis and fibrosis, and acute and chronic pancreatitis \(^\text{(14)}\). Moreover, alcohol consumption is a major cause of several types of injuries (ranging from homicide to traffic accidents) and drinking of alcohol during pregnancy is associated with various adverse effects including fetal alcohol syndrome, spontaneous abortion, low birth-weight, premature birth, and intrauterine growth retardation \(^\text{(14)}\). However, it is very important to note that evidence suggests that moderate alcohol consumption of up to two drinks per day, reduces the risk of heart disease and stroke \(^\text{(14, 15)}\).

Evidence suggests that genetic susceptibility also plays an important part in alcohol-related cancer. Alcohol dehydrogenase (ADH), a major alcohol metabolizing enzyme, oxidizes ethanol to acetaldehyde, and aldehyde dehydrogenase (ALDH) detoxifies acetaldehyde to acetate. The variant allele ALDH\(^*2\), which encodes an inactive subunit of the enzyme ALDH\(^2\), is dominant and highly prevalent in certain eastern-Asian populations (28-45\%), but rare in other ethnic groups. Most homozygous
carriers of this allele (ALDH2*2/*2) are abstainers or infrequent drinkers, mainly because the enzyme deficiency causes strong facial flushing, physical discomfort and severe toxic reactions. In heterozygous carriers (ALDH2*1/*2) these acute adverse effects are less severe but when this population consumes alcohol they are at higher risk for severe alcohol-related aero-digestive cancers. For example, genetic epidemiological studies provide strong evidence that the heterozygous ALDH2*1/*2 genotype contributes substantially to the development of esophageal cancer related to alcohol consumption with relative risks as high as 12 for heavy drinkers(16). Evidence also suggests that the disease effect of alcohol is modulated by polymorphisms in genes encoding enzymes for ethanol metabolism (alcohol dehydrogenases (ADH) and cytochrome P450 2E1), folate metabolism and DNA repair (14).

Nutrition may also play a large role in the development of alcohol-induced liver injury. Nutritional status depends on access to food and other cultural and behavioral food utilization patterns. Both experimental and epidemiological studies have demonstrated that chronic ethanol ingestion causes depletion of nutrients such as choline, folate and methionine (17). This depletion of nutrients is thought to be caused by malnutrition, decreased absorption of nutrients, and toxic interaction of ethanol with nutrient metabolism (17). Moreover, the synergistic interaction between nutritional deficiency and ethanol results in exacerbated liver injury (18). With this being said, malnutrition can play a major role in the pathogenesis of chronic alcohol exposure.

Overall, the linear dose-response relationship between alcohol intake and risk of disease shows that control of heavy drinking remains the main target for alcohol-induced disease control. Patients with cirrhosis and HCC are generally asymptomatic in the early stages of their disease. For this reason they are usually diagnosed at late stages of
development where the disease is progressing rapidly, is poorly responsive to non-operative therapy and also has a low rate of resectability; therefore, prevention is the key to reducing morbidity and mortality caused by cirrhosis and HCC (19). Moreover, in many countries, people of low income or low education consume more alcohol compared to those of high income and education, which contributes to social inequalities in alcohol-related disease burden (14). Total abstinence of alcohol, although optimal for cancer control, may not be the best solution in terms of a public health perspective, especially in countries and regions with high incidence of cardiovascular disease.

**Alcohol Metabolism**

Alcohol is processed in the body through various metabolic pathways, producing toxic byproducts that contribute to cell and tissue damage. Moreover, mechanisms of ethanol-induced liver injury and carcinogenesis are closely related to the metabolism of ethanol. When alcohol is ingested, it is absorbed directly from the stomach and small intestine and subsequently distributed throughout the entire fluid space of the body. After equilibrium is reached, alcohol will be found in all tissues of the body in proportion to their water content. Between 95-98% of the total amount of alcohol consumed is metabolized by enzymatic oxidation in the liver. The remaining 2-5% of the dose of alcohol consumed is excreted unchanged in urine, sweat, and expired air (20).

Ethanol is metabolized to acetaldehyde by three main mechanisms; alcohol dehydrogenase (ADH), cytochrome P450 (CYP) 2E1, and catalase. ADH-mediated ethanol metabolism in cytosol metabolizes ethanol to acetaldehyde by utilizing the cofactor nicotinamide adenine dinucleotide (oxidized form, NAD+) and converting it to a reduced form, known as NADH.
After moderate alcohol consumption, most ingested alcohol is broken down by the ADH pathway; however, after chronic heavy alcohol consumption the microsomal ethanol-oxidizing system (MEOS) system can play a role. The primary component of MEOS is cytochrome P450, which exist in multiple families and subfamilies. The most important subfamily for alcohol metabolism is cytochrome P450 2E1 (CYP2E1). Electrons are transferred from nicotinamide adenine dinucleotide phosphate (NADPH) to CYP2E1 by cytochrome P450 reductase. Alcohol metabolism by CYP2E1 generates several types of highly reactive oxygen-containing molecules known as reactive oxygen species (ROS) (21). Thus, alcohol metabolism through MEOS can lead to liver damage both by generating ROS and by reducing glutathione levels (22).

Catalase, found in peroxisomes, has also been shown to participate in the metabolism of alcohol. Quantitative assessments have indicated that the at ethanol concentrations ranging from 0.5 to 30 mM the predominant ethanol oxidation pathway is alcohol dehydrogenase-dependent; however, at higher ethanol concentrations, a significant portion of total ethanol metabolism, up to 50%, is mediated by catalase-hydrogen peroxide complex (23). It was found that rates of oxygen uptake after alcohol or methanol treatment in vivo are identical in isolated perfused liver, supporting the suggestion that catalase plays an important role in Swift Increase of Alcohol Metabolism (SIAM) (24).

**Mechanisms of Alcohol-induced liver damage**

Alcoholic hepatitis and other forms of alcoholic liver disease (ALD) are major complications of chronic excessive ethanol intake, which generally amounts to many years of daily alcohol use in excess of 100 g/day. Both clinical findings and animal
models of alcoholic hepatitis have shown the importance of cytokine mediated cell–cell interactions in the onset of ethanol-induced liver damage. Kupffer cells activated by gut-derived endotoxin (LPS) may play an important role in the mechanism of early alcohol-induced liver injury (ALI) (25). Activation of Kupffer cells, which is critical for producing a hypermetabolic state (SIAM), results from exposure to endotoxin that derives from the cell wall material of Gram-negative bacteria in the gut (26). Elevated levels of circulating endotoxin have been detected in heavy drinkers (27), as well as in rats after chronic ethanol administration (28). Kupffer cells respond to the endotoxin challenge by producing a battery of cytokines and chemokines, including TNF-α, interleukin (IL)-1β, IL-6, and prostaglandin E₂ (26, 29). Inactivation of Kupffer cells with gadolinium chloride diminished free radical formation and prevented early ALI (30). Furthermore, intestinal sterilization with antibiotics diminished endotoxin and minimized ALI in rats (31).

Ethanol administration in vivo is associated with the formation of free radicals due to oxidant stress (32). Reactive oxygen species (ROS) and reactive nitrogen species (RNS), generated in response to cytokine-induced stress signals in parenchymal cells and also by activation of Kupffer cells and inflammatory cells, further mobilize cellular defense mechanisms. Cytokines and chemokines released by Kupffer cells exert a range of autocrine and paracrine effects that initiate defense responses in the liver, but also promote infiltration of inflammatory leukocytes and activate oxidative responses, accompanied by a further release of cytokines and degradative proteins. These complex cascades result in a vicious cycle of acute challenges to liver cells by oxidative stress and cytotoxic signals that can overwhelm cellular defense mechanisms (33).
Cytochrome P450 (CYP) 2E1 is induced predominantly in the hepatocytes by ethanol and could also be a source of reactive oxygen species which could lead to liver injury (34). A correlation has been shown between blood levels of alcohol and induction of CYP2E1 as alcohol cycles in the enteral model of continuous alcohol administration. Notably, the level of CYP2E1 correlates with the degree of pathology (35). Inhibitors of CYP2E1 partially reduced hepatic pathology caused by enteral ethanol feeding (35). Fatty liver and accumulation of triglyceride in liver were observed in wild-type (CYP2E1 +/+) mice but not in CYP2E1-knockout (CYP2E1 -/-) mice. Interestingly, the introduction of CYP2E1 to CYP2E1 knockout mice via an adenovirus restored macrovesicular fat accumulation (36). However, Kono et al. showed that there was no difference in pathology between CYP2E1 wildtype and CYP2E1 knockout mice treated with ethanol over 4 weeks (25). This suggests oxidants from CYP2E1 play only a small role in mechanism of early ALI in mice.

Acetaldehyde, a product of ethanol metabolism, is labeled as a carcinogen in animals (37). Acetaldehyde may be the important cancer-causing agent in the upper and lower gastrointestinal tract because acetaldehyde concentrations in saliva and the large intestine are high enough to enable it to act as a carcinogen (38). Acetaldehyde also binds to proteins, resulting in structural and functional alterations. Acetaldehyde concentrations in the liver are significantly lower due to an effective acetaldehyde metabolizing system, therefore oxidative stress and other factors may be more important. Conversely, acetaldehyde has been shown to play a role in preventing liver pathology by preventing NFκB activation (39).

Malnutrition can play a role in alcohol-induced liver injury and is a common finding in chronic alcoholics. These patients also demonstrate frequent deficiencies of
folate, thiamine, pyridoxine, and vitamin A, which enhance the likelihood of anemia, altered cognitive states, and night blindness. The etiologies of malnutrition in ALD patients are multiple and interactive and include anorexia with inadequate dietary intake, abnormal digestion of macronutrients and absorption of several micronutrients, increased skeletal and visceral protein catabolism, and abnormal interactions of ethanol and lipid metabolism (40).

**Tsukamoto-French Model**

The establishment of a continuous intragastric enteral feeding protocol in rodents by Tsukamoto and French was a major development in research in ALI (41). This model of alcoholic liver disease (ALD) has been utilized in various laboratories to study various aspects of ALD pathogenesis including oxidative stress, cytokine upregulation, hypoxic damage, apoptosis, ubiquitin-proteasome pathway and CYP2E1 induction (39). The basic value of the model is that it produces pathologic changes which resemble human ALD including microvesicular and macrovesicular fat, megamitochondria, apoptosis, central lobular and pericellular fibrosis, portal fibrosis, bridging fibrosis, central necrosis, and mixed inflammatory infiltrate including polymorphonuclear leukocytes (PMNs) and lymphocytes (42). The model is valuable because the diet and ethanol intake are totally under the control of the investigator. In any case, this model has revolutionized our understanding of the pathogenesis of alcohol-induced liver disease.

One of the most interesting phenomenon discovered using the Tsukamoto-French model was the fact that chronic ethanol feeding at a constant rate causes a cyclic oscillation in the blood and urine alcohol levels. A steady state can be maintained with high or low blood alcohol levels for long periods. The cycling of the blood alcohol levels,
when a constant infusion rate of alcohol is maintained, simulates binge drinking (39). The blood alcohol level cycle (BALC) of the intragastric tube feeding model first described by Tsukamoto et al., has three separate essential mechanistic components. The first is the requirement for an intact functioning thyroid. The evidence for this is that severance of the pituitary stalk completely prevents the cycle. What happens instead of the cycle is that the blood alcohol level rises to a lethal level when ethanol is given continuously at a dose of 11 g/kg/day by stomach tube (43). When excess thyroid hormone is given orally it markedly attenuates the cycle because it interferes with the changes in the level of thyroid hormone during the cycle.

The second component of BALC is norepinephrine. Catecholamines are markedly elevated at the peaks of the cycle. Both propranolol and phenoxybenzamine, which are beta- and alpha-blockers, prevent the cycle. Also, when catecholamines are fed in excess in the form of ephedrine, the cycle is eliminated (39). The third element essential to the cycle is the generation of NAD to support the oxidation of alcohol by alcohol dehydrogenase. When complex I (NADH dehydrogenase) of the mitochondrial electron transport chain is inhibited by feeding rotenone, the cycle is totally eliminated and blood alcohol levels remain constant at 200 mg/100 ml. Thus NADH increases and NAD decreases at the peak of the cycle. Without the fluctuation of NAD, ADH activity cannot fluctuate during the cycle and the cycle is prevented. At the BALC peaks there is increased liver pathology, especially inflammatory changes in the liver associated with an increase of iNOS expression (39). The NADH/NAD ratio is markedly increased and ATP levels are markedly decreased at the BAL peaks. Also, endotoxin in the blood is very high at the peaks and very low at the troughs.
When the blood alcohol levels fall during the cycle, there is an increase in ALT, suggesting that reoxygenation from the hypoxic state at the peaks causes an ischemic reperfusion injury-like lesion in the liver (39). These contrasting findings at the peaks and troughs indicate that the blood alcohol levels, which fluctuate up and down, change the gene expression and the pathology of the liver. When rats are fed ethanol intragastrically, urine alcohol concentrations are low when body temperature, oxygen consumption and circulating hormones (T₄) are high (43).

*Methionine Metabolism Cycle*

Methionine is an essential amino acid that cannot be synthesized by mammals and must be obtained through food sources (e.g. plants). Mammals cannot synthesize methionine they can still utilize it in a variety of biochemical pathways. The first step in methionine metabolism is the formation of S-adenosylmethionine (SAM) in a reaction catalyzed by methionine adenosyl-transferase (MAT) (44, 45). Under normal conditions, most of the SAM generated is used in trans-methylation reactions, where SAM is converted to SAH by transferring the methyl group to diverse biological acceptors (44). SAH is then converted to homocysteine and adenosine in a reversible reaction catalyzed by SAH hydrolase (44). In the liver, homocysteine is metabolized by trans-sulfuration and trans-methylation pathways.

In the trans-sulfuration pathway, homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine β-synthase, which requires vitamin B-6 (pyridoxine) as a cofactor. The activity of cystathionine β-synthase is allosterically regulated by SAM (46, 47). Cystationine is then cleaved by another B-6 dependent
enzyme, γ-cystathionase, which results in the release of free cysteine, the rate-limiting precursor for reduced glutathione synthesis (44, 48).

In the trans-methylation pathways, homocysteine can be converted to methionine by two separate reactions (44, 45). Normally, utilizing folate and through the action of methionine synthetase (MS), a methyl group is transferred from N5-methyltetrahydrofolate (MTHF) to vitamin B-12 to form methyl-cobalamine, which in turn transfers the methyl group to homocysteine to produce methionine (49). When MS is compromised by exposure to ethanol a methyl group is transferred from betaine to homocysteine to from methionine in a reaction catalyzed by betaine-homocysteine methyltransferase (BHMT) (17, 50, 51). The conversion of homocysteine to methionine is an essential reaction to conserve methionine, detoxify homocysteine, and produce SAM (49).

**Methyl Deficiency**

Experiments of rodent methyl deficiency in vivo have shown that such diets lead to rapid fat accumulation in the liver, increased lipid peroxidation, necrotic and apoptotic cell death, increased cell proliferation, depletion of intracellular methyl group pools, and hypomethylation of hepatic DNA (52-54). After nine weeks of methyl deficient diet, levels of hepatic SAM in rats were decreased by 70% (52). Exposure of animals to methyl-deficient diet for longer periods did not lead to further reduction of liver SAM content. (52). On the other hand, methyl-deficient diet did not change SAH levels in rat liver. (52). Methyl-adequate diet has the ability to restore liver SAM levels in rats fed methyl-deficient diet for long periods of time; however, re-feeding the methyl-adequate diet restored DNA methylation to normal level only in the group that had been fed
methyl-deficient diet for 9 weeks; in animals exposed to methyl deprivation for longer periods of time, the methyl-adequate diet failed to reverse the hypomethylation of DNA, which has been associated with increased risk for tumorigenesis (52).

**Role of SAM in alcohol-induced liver injury**

SAM, a metabolite of methionine, is an important molecule that is required for many vital functions and survival of cells in the body. It is the principal biological methyl donor required for methylation of DNA, RNA, biogenic amines, phospholipids, histones, and other proteins (49). It is a precursor for the synthesis of polyamines, which are required for cell proliferation and the maintenance of cell viability. In the liver, SAM participates in the synthesis of GSH, a major endogenous antioxidant that protects cells against toxins like free radicals (55). SAM synthesis and metabolism take place predominantly in the liver. Up to 85% of all methylation reaction and as much as 48% of methionine metabolism occur in the liver (56). SAM deficiency can impair many vital functions in the liver, which render it susceptible to injury by toxic agents such as alcohol (49).

Both animal and human studies suggest a relationship between ethanol consumption and hepatic SAM depletion. Chronic ethanol administration depleted hepatic concentrations of SAM in rats (57-59), mice (60), baboons (61) and micropigs (50). Reduced hepatic SAM concentrations have also been reported in alcoholic hepatitis patients (62). Hepatic SAM depletion by chronic ethanol administration is associated with liver injury of variable magnitude: fatty liver in rats (57), fatty liver, inflammation, and fibrosis in baboons (61), fatty liver and inflammation in micropigs (63) and hepatitis in humans (62). The effect of SAM depletion is well characterized in methionine
adenosyl-transferase (MAT)1A knockout mice, which have markedly elevated serum methionine concentrations and reduced hepatic SAM and GSH concentrations (64). At three months, MAT1A knockout mice develop hepatic hyperplasia and are more prone to develop fatty liver due to a choline deficient diet. At eight months of age, these mice spontaneously develop NASH (65) and by 18 months the majority of the knockout mice develop hepatocellular carcinoma even when consuming a normal diet (66).

Several mechanisms have been proposed for SAM depletion including inactivation of MAT, excessive consumption of SAM by liver, and inhibition of endogenous methionine synthesis due to impaired homocysteine methylation. Inactivation of MAT impairs the metabolism of methionine to SAM, leading to SAM depletion as seen in MAT1A knockout mice (65). Chronic alcohol exposure may decrease hepatic SAM concentrations by inactivating MAT. The activity of MAT was significantly reduced in liver biopsy from alcoholic and non-alcoholic cirrhotic patients (67, 68). This effect could be partly due to a decreased expression of MAT. Recent data indicate a 50% decrease in the expression of MAT1A (liver specific) in liver sample obtained from alcoholic hepatitis patients and from ethanol-fed micropigs (62, 69). MAT1A expression is diminished in end-stage cirrhotic patients independent of etiology of disease (70).

Ethanol metabolism is known to generate free radicals, both reactive oxygen species and reactive nitrogen species, which may inactivate hepatic MAT through the oxidation or nitrosylation of a critical cysteine residue at position 121 (45, 49). Ethanol may deplete hepatic SAM concentrations by increased SAM consumption. This was apparent in a rat study in which chronic alcohol administration decreased hepatic SAM and glutathione concentrations without affecting MAT activity (71). This suggests that
the utilization of SAM is increased as the precursor for the synthesis of GSH to counteract alcohol induced oxidative stress (49).

The fact that SAM is a key factor for a multitude of metabolic reactions coupled with the fact that chronic ethanol consumption can deplete its hepatic concentrations in association with liver injury has prompted many investigators to evaluate its role in the attenuation of ALI. SAM administration attenuated alcohol-induced steatosis and restored hepatic GSH concentrations in rats (72). In mice, SAM treatment significantly attenuated steatosis, necrosis, and serum ALT levels (60). In baboons, SAM attenuated ALI by repairing mitochondrial injury, which thereby restored plasma GSH concentrations and decreased plasma AST concentrations (61).

The therapeutic potential of SAM was tested in a 24-month randomized, placebo-controlled, double-blind, multi-center clinical trail in patients with alcoholic cirrhosis in Spain. SAM treatment improved survival or delayed the need for liver transplantation in patients with alcoholic liver cirrhosis, especially in those with less advanced liver disease (73). Increased concentrations of GSH may have played a role in the beneficial effect of SAM in the latter study because in a subsequent study hepatic GSH concentrations were significantly elevated in ALD patients after oral administration of 1.2g SAM/day for 6 months (74). However, a recent clinical meta-analysis could not find evidence to support or refute the efficacy of SAM in the treatment of patients with ALD (75). It is important to note that SAM has also been shown to attenuate non-alcoholic induced liver injury. In rats, SAM treatment attenuated CCl4-induced liver fibrosis by restoring hepatic MAT activity and glutathione concentrations and by reducing lipid peroxidation (76). In acetaminophen-treated mice, SAM significantly attenuated liver injury by preventing
decreases in liver and blood SAM concentrations and by attenuating both cytosolic and mitochondrial glutathione depletion and mitochondrial dysfunction (77).

SAM may provide protection against liver injury by the following mechanisms: attenuation of oxidative stress by restoring glutathione concentrations, attenuation of inflammation, prevention of apoptosis of hepatocytes and/or induction of apoptosis of liver tumor cells. Oxidative stress plays a major role in the development of alcoholic liver injury. This can occur because of excess accumulation of free radicals, delayed elimination of free radicals or a combination of the two (49). Free radicals are atomic or molecular species with unpaired electrons. GSH is capable of attenuating oxidative stress by scavenging free radicals such as superoxide (O$_2^-$) and hydroxyl radicals (-OH). One way to attenuate alcoholic liver injury is to increase hepatic concentrations of glutathione.

SAM administration restored hepatic concentrations of glutathione depleted by alcohol in rodents and patients affected with ALD and non-ALD (60, 74). It is also important to note that SAM can restore hepatic mitochondrial glutathione concentration which is essential for the maintenance of mitochondrial function and protection from free radical damage (78). Cytokines play an important role in both the initiation and the attenuation of ALI. SAM may attenuate inflammation and therefore ALD, by down-regulating tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, and up-regulating interleukin-10 (IL-10), an anti-inflammatory cytokine (79, 80). Apoptosis is a form of programmed cell death. Some research has also shown that SAM has the ability to prevent apoptosis of hepatocytes. SAM and its metabolite, MTA, protected rat hepatocytes from okadaic acid-induced apoptosis in a dose-dependent manner. Moreover, SAM has been shown to induce apoptosis in liver cancer cell lines (81). In
other words, SAM has the ability to inhibit apoptosis of normal cells yet stimulate apoptosis of liver cancer cells.

**Role of folate in alcoholic liver disease**

Folate is a water-soluble vitamin that plays an integral role in methionine metabolism and helps maintain normal concentrations of homocysteine, methionine, and SAM. Folate deficiency can impair methionine metabolism, leading to increased levels of homocysteine and depletion of methionine and SAM (40). Decreased serum folate concentrations have been reported in the majority of chronic alcoholics who consume more than 80 g of ethanol per day (82). The possible causes of folate depletion in alcoholics include diet lacking in folate-rich foods (82), malabsorption in the intestine (83), decreased liver uptake (84), and increased urinary excretion of folic acid (85).

Increasing evidence suggests that homocysteine is involved in the pathogenesis of alcoholic liver injury (86). Folate is required for metabolism of homocysteine, and therefore its deficiency can lead to elevations of serum homocysteine concentrations. In addition to depleting folate concentrations, chronic ethanol exposure decreases the activity of methionine synthase (MS) which is required to catalyze the transfer of the methyl group from folate to homocysteine in order to form methionine (50). Therefore, chronic ethanol exposure has the ability to elevate plasma concentrations of homocysteine by affecting both folate concentrations and MS activity. Studies in the micropig model have shown that the onset of ALD is accelerated in the presence of folate deficiency, which also amplifies the effect of chronic ethanol exposure on altered methionine metabolism (87). Since the American diet is folic acid (synthetic folate) fortified, chronic exposure to ethanol may not deplete hepatic folate to an extent
sufficient to impair homocysteine metabolism, although ethanol may still impair homocysteine metabolism by inhibiting the activity of MS ((88). Folate deficiency alone does not lead to liver injury, but it can exacerbate or possibly promote the development of ALD. It is not known if exogenous folate administration can attenuate ALD.

**Role of betaine in alcoholic liver disease**

Dietary betaine can be absorbed from the intestine and transported to the liver. Betaine has the ability, in the liver, to transfer its one methyl group to homocysteine in order to form methionine. For this reason, one can expect decreased concentrations of homocysteine and increased concentrations of methionine, inherently leading to an elevation in the SAM:SAH ratio. Interestingly, the SAM:SAH ratio can trigger a cascade that restores phosphatidylethanolamine methyltransferase (PEMT) activity, elevates phosphatidylcholine (PC) concentrations, and normalizes VLDL (very low density lipoproteins) which is required for the export of liver triacylglycerol (89). The cascade essentially leads to attenuation of fatty liver.

The intragastric ethanol-fed model of alcoholic liver injury was used to further understand the role of betaine in the treatment of ALD. Betaine feeding attenuated the pathologic features of ALI (fatty liver, necroinflammation and apoptosis) and also decreased plasma ALT concentrations (90). These features were associated with attenuated plasma homocysteine concentrations, increased hepatic SAH concentrations, an increase in SAM concentration, and several fold increased SAM:SAH ratios. In addition, betaine attenuated the alcohol-induced endoplasmic reticulum (ER) stress response which was shown by the down-regulation of proapoptotic gene GADD153 and of lipogenic transcription factor SREBB-1 (90). To better understand the role of betaine
as a treatment for fibrosis, rats were used in a model that administered ethanol and carbon tetrachloride (CCL₄) in order to induce fibrosis. Betaine treatment in this model prevented liver fibrosis, attenuated lipid peroxidation, and decreased plasma ALT levels (91).

**Role of zinc in alcoholic liver disease**

Decreased concentration of zinc in the liver is one of the most consistent observations in alcoholic patients and animal models of alcoholic liver injury. The consequence of zinc depletion has not been well understood, although it has been suggested that zinc deficiency contributes to the pathogenesis of liver disease (92). During chronic ethanol exposure, both zinc and metallothionein, a metal-binding protein important in zinc homeostasis, were found to be significantly reduced; however, dietary zinc supplementation has been shown to significantly increase metallothionein concentrations in livers exposure to alcohol (93, 94). Zinc supplementation attenuated ethanol-induced liver pathology, serum ALT and hepatic TNF-α in metallothionein-knockout and wild-type 129/Sv mice, indicating a metallothionein-independent pathway of protection (95). Zinc supplementation suppressed ethanol-elevated cytochrome P450 2E1 activity but increased the activity of alcohol dehydrogenase, without affecting the rate of blood ethanol elimination. Zinc supplementation also prevented ethanol-induced decreases in glutathione concentration (96).

**Choosing the experimental diet**

Methyl supplements increase the level of DNA methylation in the agouti mouse and change the phenotype of offspring in the healthy, longer-lived direction (97). This shows that methyl supplements have strong effects on DNA methylation and phenotype
and are likely to affect long-term health. The methyl groups of 5-methyldeoxycytidine (5-MC) are either synthesized *de novo* in one-carbon metabolism or are supplied preformed in the diet. These processes rely on essential dietary folates (for one-carbon metabolism), dietary methionine or dietary/endogenous betaine and choline (preformed methyl groups) (97). Moreover, methionine, zinc, and vitamin B-12 (cobalamin) are all dietary essentials and are used as intermediates and enzymatic cofactors to transport and transfer methyl groups in methyl metabolism (98). Methyl-supplemented diets were designed to provide substantially increased amounts of cofactors and methyl donor for optimum methyl metabolism (98).
CHAPTER 2

Introduction

Alcoholic liver disease is characterized by fatty liver, steatohepatitis, fibrosis, cirrhosis and potentially hepatocellular carcinoma. Several mechanisms have been proposed for the pathogenesis of alcoholic liver disease (ALD) including acetaldehyde toxicity, oxidative stress, endotoxins, cytokines, chemokines, a compromised immune system and nutritional deficiencies (49). Increasing evidence suggests that altered methionine-folate metabolism can also contribute to the development of ALD (87, 99). Chronic ethanol exposure has been shown to decrease hepatic concentrations of S-adenosylmethionine (SAM), a product of methionine metabolism (57, 60, 61). Chronic alcohol exposure has also been shown to increase plasma concentrations of homocysteine (90, 100, 101), increase hepatic concentrations of S-adenosylhomocysteine (SAH) (17, 50, 102), and decrease plasma concentrations of folate in animals and humans (103). Exogenous SAM administration has been shown to attenuate liver injury in animals (60, 61, 72). Importantly, SAM has also been used to improve survival or delay the need for transplantation in patients with alcoholic liver cirrhosis (73).

Betaine, a metabolite of choline and an important factor in methionine metabolism, has also been shown to attenuate alcoholic liver injury by increasing the concentrations of hepatic SAM and decreasing homocysteine and SAH in animal studies. (51, 90, 102, 104). SAM and betaine supplementation in the chronic ethanol model have been shown to increase SAM concentrations and the SAM:SAH ratio, which has also been shown to play a role in the regulation of cellular glutathione (GSH) which is vital to cellular antioxidant defense (72, 90). Understanding the role of SAM, folate and betaine in mitigating alcoholic liver injury may help to develop effective and safe therapies for
alcohol-induced liver injury (ALI) and non-ALI. By utilizing an array of methyl donors and co-factors including methionine, choline, betaine, folate, vitamin B12, and zinc, we have designed a model system that permits sufficient methionine-folate metabolism during chronic alcohol administration and therefore prevents alcohol-induced liver injury.
CHAPTER 3
Materials and Methods

Animals
Male C57BL/6J mice (22-26 g body weight, 8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All animals were housed in pathogen-free barrier facilities accredited by the AAALAC, and procedures used were approved by the local IACUC.

Diets
Animals were randomly divided into two groups and were fed either standard Isopro RMH 3000 rodent diet (Lab Diet, Richmond, IN) or methyl supplemented pellet diet – TD.01310 –Custom Rodent Diet 7017, 3MS, ZM (Harlan-Teklad, Madison, WI) for 1 week prior to surgery. The methyl pellet diet was designed to provide substantially increased amounts of cofactors and methyl donors (105). A basic high fat liquid diet (HFD) was prepared according to Thompson and Reitz (106) as described previously and supplemented with lipotropes as described by Morimoto et al. (107). Control HFD (1.3 kcal/ml) contained corn oil as fat (37% of total calories), protein (23%), carbohydrate (40%), plus vitamins and minerals. Isoalcoric ethanol diet was prepared by replacing maltose-dextrin with ethanol (108). Methyl donor enriched high fat diet (MDHFD) was prepared by fortifying basic HFD (mentioned above) with 15 g/kg betaine, 15 g/kg choline chloride, 15 mg/kg folic acid, 7.5 g/kg L-methionine, 0.15 mg/kg vitamin B12 and 660 mg/kg zinc sulfate, which is the same methyl donor and cofactor regimen found in the 3MS, ZM pellet diet (Refer to Table 1).
**Surgery**

The surgical procedures used here were similar to methods described previously by Tsukamoto and French for rats (41), with modifications to accommodate the smaller size of mice (108). Briefly, mice were anesthetized by injection of pentobarbital sodium (Abbott Laboratories, North Chicago, IL). A PE90 polyethylene tube (Becton Dickinson, Sparks, MD) was placed in the squamous area of the stomach, anchored to the stomach wall with 7-0 polypropylene sutures (Ethicon) and Dacron felt, and fixed to the abdominal wall. The tube was then tunneled subcutaneously to the dorsal aspect of the neck using an InSyte Autoguard shielded catheter (Becton Dickinson, Sandy, UT). After 0.1 mL of ampicillin was applied to the abdominal cavity, the abdominal wall was closed using 4-0 silk (Ethicon) and the abdominal skin was closed using 7mm stainless steel wound clips (CellPoint Scientific Inc., Gaithersburg, MD). The tube was passed through dorsal muscle using a 20G 1 1/2 needle (Becton Dickinson, Franklin Lakes, NJ) and the tube was anchored to the dorsal muscle using 5-0 prolene (Ethicon). A small piece of Dacron felt was threaded down the tube and rested on the dorsal muscle. After 0.1 mL of ampicillin was administered to the open wound, the cervical-dorsal skin was closed using 4-0 silk. An empty 1 mL syringe was threaded over the tube to keep the mouse from biting the tube. The feeding tube was attached to an infusion pump in order to continuously deliver the liquid diet. Mice had complete mobility inside the metabolic cage. Gentamicin (0.125mL) was administered subcutaneously two times per day for three days post-surgically.
Experimental protocol

After surgery, animals were placed in individual metabolic cages. All animals had free access to water. Animals in the HFD groups had free access to Isopro RMH 3000 pellet diet; whereas, animals in the MDHFD groups had free access to 3MS, ZM pellet diet. Liquid diets were initiated one week after surgery to allow for full recovery. The liquid diet was fed continuously at the rate of 9-11 ml/day to achieve weight gain. Behavior was assessed using a 0 to 3 scoring system (0, normal; 1, sluggish movement; 2, loss movement but still moving if stimulated; 3, loss of consciousness). Based on the score, alcohol administration was then adjusted carefully to prevent overdosing. Ethanol initially was delivered at 17.3 g/kg per day and was increased 1.3 g/kg per 2 days until day 8. The dose was 22.3 g/kg per day from days 9 to 12; 23.3 g/kg per day from days 13 to 20; 24.2 g/kg per day from days 21 to 24; 25.6 g/kg per day from days 25 to 26; and a final dose of 27 g/kg per day on days 27 and 28. Mice were sacrificed after 4 weeks of treatment and blood samples were collected via the inferior vena cava at necropsy. Blood was centrifuged and serum was collected in microtubes and stored at -80°C. Livers were excised and weighed and tissue samples were divided; some were formalin-fixed, embedded in paraffin, cut in 5 μm sections, and stained hematoxylin and eosin (H&E) to access steatosis, inflammation, and necrosis; others were snap frozen in liquid nitrogen and stored at -80°C.
Pathological evaluation

H&E slides were scored by a single pathologist in a blinded manner as described by Nanji et al (28) as follows: steatosis (the percentage of liver sells containing fat): < 25% = 1+, < 50% = 2+, <75% = 3+, > 75% = 4+; inflammation and necrosis: 1 focus per low-power field = 1+, 2 or more foci = 2+.

Clinical Chemistry

Concentrations of ethanol in urine are representative of blood alcohol levels (109). Mice were housed in metabolic cages that separated urine from feces and urine samples were collected over 24 h in vials containing mineral oil to prevent evaporation. Each day at 0900 h, urine collection vials were changed and a 1 ml sample of urine was stored at -20°C until it could be assayed for ethanol concentration. Ethanol levels in urine, diluted 1:20 with distilled water, were determined daily by measuring absorbance at 366 nm resulting from the reduction of NAD⁺ by alcohol dehydrogenase (110). Serum alcohol concentrations (BAC) at sacrifice were determined by diluting serum 1:20 using distilled water and measuring absorbance at 366 nm resulting from the reduction of NAD⁺ by alcohol dehydrogenase (110). To determine liver alcohol concentration, 25 mg of extracted liver tissue was homogenized in 600 µl PBS and centrifuged for 5 min at 13.2 rpm. Liver alcohol concentration was determined using supernatant of liver homogenate, diluted 1:1 with distilled water, by the previously mentioned alcohol determination method. Protein concentration of liver homogenate was determined by using the BCA Protein assay kit (Pierce Rockford, IL). Serum alanine transaminase (ALT) levels, a liver-specific surrogate marker of liver injury, were determined with Thermo Scientific Infinity ALT Liquid stable reagent (Product # TR71121) using the rates of change in
absorbance at 340 nm at 37°C and the molar extinction coefficient for NADH to calculate activities.

Administration of BrdU

BrdU, 5-bromo-2'-deoxyuridine, is often used as a marker of cell proliferation. A BrdU stock solution, 10 mg/ml, was prepared and BrdU stock solution was administered on days 25 through 28 by adding 0.02 ml/g of liquid diet.

Immunohistochemical Detection of 5-bromo-2'-deoxyuridine (BrdU)

Formalin-fixed, paraffin-embedded liver sections (6 μm) were mounted on glass slides. Sections were deparaffinized in xylene, rehydrated in a series of graded alcohol concentrations, and placed in PBS with 1% Tween 20. Immunostaining was performed using DAKO EnVision System HRP (Dako Cytomation, Carpinteria, CA) with primary Monoclonal antibody BrdU (DAKO, clone Bu20a) diluted in PBS containing 1% bovine serum albumin and incubated overnight at 4°C. Slides were counterstained with hematoxylin. In order to ensure the quantitative measurement of each immunoreaction, all sections from each animal and group to be compared were processed in parallel. Quantitative analysis of immunostained liver sections was performed by averaging percent positively stained nuclei to total nuclei within 10 random fields at 200×.

ADH activity

The ADH activity assay was modified from a method described by Kathy Crow (111). Liver extracts were homogenized in 10 volumes of 0.05M HEPES pH 8.4 and 0.33mM dithiothreitol. Samples were centrifuged at max speed (13,600 x g) for 30 minutes at 4°C and 0.02 ml of supernatant was added to an assay mixture containing 0.1 ml 1M Tris
Base (pH 7.2), 0.02 ml of 28 mM NAD+, 0.01 ml of 0.1M ethanol, and 0.05 ml DI water and was incubated at 37°C and the linear decline in OD at 340 nm was determined for 6-10 min. Enzyme activity was calculated based on change in OD/ mg protein/ min using an extinction coefficient of 6.22 μmol/ml/ 0.5 cm. Protein concentration of supernatant was determined by using the BCA Protein assay kit (Pierce Rockford, IL).

**CYP2E1 Activity**

To obtain S9 supernatant fraction, liver samples were homogenized in 10 mM Tris, 0.25 M sucrose, 1 mM EDTA and then spun for 20 minutes at 10,000 x g at 4°C. S9 samples were stored at -80°C, thawed on ice before use and kept on ice at all times. Activity of CYP2E1 in S9 fractions was assayed by measuring the hydroxylation of p-nitrophenol to p-nitrocatechol (PNP) (112).

**Catalase Activity**

Catalase activity was measured using Caymen catalase assay kit (Product # 707002) (Caymen Chemical Company, Ann Arbor, MI). Stomach samples for stomach catalase activity was diluted 1:50 using assay buffer, whereas, liver samples for liver catalase activity was diluted 1:2000 using assay buffer.

**Acyl CoA Oxidase Activity**

Acyl CoA oxidase is a peroxisomal enzyme which generates H₂O₂. Importantly, catalase utilizes H₂O₂ to metabolize alcohol to acetaldehyde. Peroxisomal oxidation of palmitate was used to estimate acyl CoA oxidase activity. Liver samples (~100 μg) were homogenized in 10 volumes of ice cold 50 mM Tris-HCl and 0.25 M sucrose, pH 7.2. Homogenates were then frozen overnight at -80°C. Homogenates were thawed and then
centrifuged at 13,600 x g for 30 min at 4°C to obtain supernatant. Supernatant was incubated at 37°C with a reaction mixture containing (in mg) 13.9 palmitate, 3.54 CoA, 68.9 ATP, 20.4 MgCl₂, 66.7 NAD⁺, 45 fatty acid free albumin, 36.8 semicarbazide, 370 Tris base, 307 Tris HCl, and 201 niacinamide, and 200 µl methanol, 50 ml DI water and 5 µl Triton X-100 in order to generate formaldehyde from methanol. Formaldehyde concentration was determined using NASH reagent (5 g ammonium acetate, 40 µl acetyl acetone, and 10 ml DI water) at absorbance 405 nm (ε = 6.58) (113). Under these conditions, formaldehyde concentration is equivalent to H₂O₂ concentration.

**Determination of Aminothiols**

The determination of glutathione [reduced] (GSH), glutathione [oxidized] (GSSG), cysteine, cysteinylglycine (Cys-Gly), and glutaminecysteine (Glu-Cys) content in liver tissue extracts was performed by HPLC using coulometric electrochemical detection as previously described (114).

**Statistics**

One-way ANOVA followed by a Tukey’s post-hoc test was used for the determination of statistical significance as appropriate. For comparison of pathological scores, the Mann-Whitney U rank sum test was used. Data are presented as mean ± SEM. A p < 0.05 was selected before the study as the level of significance.
CHAPTER 4

Results

Body weight gain

Steady weight gains were obtained during 4 weeks of continuous enteral feeding of high fat and methyl enriched high fat liquid diets, with or without ethanol, indicating adequate nutrition. There were no significant differences in weight gain among the groups studied.

Mean daily UAC

Urine alcohol concentrations (UAC) were monitored to index the degree of intoxication. As reported previously in studies with C57BL/6J mice (108) and rats (115), urine ethanol levels fluctuated in the mice fed enteral ethanol diet. Mice fed HFD containing ethanol had typical UAC cycle patterns (30 to 550 mg/dl); whereas UAC cycles in the mice fed MDHFD containing ethanol were severely blunted (10 to 350 mg/dl) (Fig.1A). The cyclic pattern observed is a phenomenon shown to be caused by hormones from the hypothalamic-pituitary-thyroid axis (43). Mean daily UAC for mice fed MDHFD containing ethanol was significantly lower, 123 ± 17 mg/dl, than mice fed HFD containing ethanol, 225 ± 34 mg/dl, even though both groups were administered diets with analogous ethanol concentrations (See Figure 1B and Table 2).

Pathological evaluation

Figure 2 shows representative photomicrographs of livers from mice fed HFD control and ethanol and MDHFD control and ethanol after 4 weeks of enteral feeding. Figure 2 also shows a photomicrograph representing naïve liver. There was no steatosis seen in either control group (Fig. 4, A and C and Table 2). Moderate fatty accumulation was observed
in mice fed HFD containing ethanol with a typical pattern of massive large droplets of fat; whereas, mice fed MDHFD containing ethanol had significantly less steatosis (Fig. 4, B and D and Table 2). Inflammation was observed in all treatment groups and also in naïve animals. Necrosis was seen in both control and ethanol HFD groups; whereas, no necrosis was observed in either control or ethanol MDHFD groups (Table 2). Mice fed HFD containing ethanol had a total pathology score of 5.6 ± 0.5 which was significantly higher than all other groups (Fig. 2 F and Table 2).

*Serum transaminase*

Serum ALT levels were 16 ± 4 U/L after 4 weeks in mice HFD (Table 2). Four weeks of enteral ethanol treatment significantly increased serum ALT in mice fed HFD containing ethanol (76 ± 26 U/L) compared to HFD control animals. Although not statistically significant using our conservative parameters, serum ALT levels were greatly reduced in mice fed MDHFD containing ethanol (28 ± 7 U/L) as compared to mice fed HFD containing ethanol.

*BAC, LAC & UAC at sacrifice*

Blood alcohol concentration (BAC) at sacrifice was significantly increased in mice fed HFD containing ethanol (194 ± 61 mg/dl) compared to mice fed HFD control (24 ± 5 mg/dl). BAC at sacrifice in mice fed MDHFD containing ethanol (239 ± 36 mg/dl) was significantly different compared to mice fed MDHFD control (30 ± 5). At sacrifice, there was no difference in BAC levels between mice fed HFD containing ethanol and those fed MDHFD containing ethanol (Table 2). There were no differences in liver alcohol concentration (LAC) at sacrifice between the two ethanol treated groups; HFD containing
ethanol (22 ± 5 μg EtOH/μg protein) and MDHFD containing ethanol (17 ± 2 μg EtOH/μg protein) (Table 2). At sacrifice, urine alcohol concentrations (UAC) were significantly elevated in mice fed HFD containing ethanol (338 ± 102 mg/dl) compared to HFD control (23 ± 2 mg/dl). Although not significant, UAC levels at sacrifice were elevated in mice fed MDHFD containing ethanol (175 ± 44 mg/dl) compared to MDHFD control (20 ± 8 mg/dl).

Liver:body weight ratios

Four weeks of enteral ethanol treatment significantly increased liver:body weight ratios in mice fed HFD containing ethanol (8.6 ± 0.4 %) compared to mice fed HFD control (6.8 ± 0.5 %), MDHFD control (6.1 ± 0.1 %), and MDHFD containing ethanol (6.0 ± 0.2 %) (Table 2). Furthermore, liver:body weight ratios in mice fed HFD control was significantly elevated compared to naïve animals (5.1 ± 0.2 %), although ratios for mice fed MDHFD, both control and ethanol, were not significantly different from naïve animals.

Hepatic cell proliferation

BrdU (5-bromo-2-deoxyuridine) is commonly used in the detection of proliferating cells. Mice fed HFD containing ethanol had significantly elevated proliferation, 10.9 ± 5.4 % BrdU, compared with all other enteral treatment groups (Table 2).
Catalase activity in the stomach and liver

Stomach catalase activity at sacrifice was significantly elevated in mice fed HFD containing ethanol (0.056 ± 0.028 mmol H₂O₂/µg protein/hr) as compared to naïve animals (0.0035 ± 0.0014 mmol H₂O₂/µg protein/hr). There was no significant difference in stomach catalase activity between the ethanol treated groups (Fig. 3). In liver, catalase activity was significantly increased in mice fed MDHFD containing ethanol (1590 ± 256 mmol H₂O₂/µg protein/hr) as compared to mice fed MDHFD (803 ± 82 mmol H₂O₂/µg protein/hr) (Fig. 4). Although not statistically significant, liver catalase was increased in mice fed MDHFD containing ethanol as compared to mice fed HFD containing ethanol (1250 ± 110 mmol H₂O₂/µg protein/hr). Moreover, catalase activity in control fed mice, HFD control (985 ± 122 mmol H₂O₂/µg protein/hr) and MDHFD control, were reduced compared to naïve animals (1370 ± 152 mmol H₂O₂/µg protein/hr).

ADH activity in the liver

Alcohol dehydrogenase (ADH) activity in the liver significantly decreased in all enterally treated animals as compared to naïve (30 ± 3 nmol NADH/mg protein/min) (Fig. 4). There was no significant difference in liver ADH activity between mice fed HFD control (8.6 ± 1.0 nmol NADH/mg protein/min) compared to HFD containing ethanol (6.1 ± 1.2 nmol NADH/mg protein/min). No significant difference was seen between mice fed MDHFD control (8.4 ± 1.4 nmol NADH/mg protein/min) and MDHFD containing ethanol (6.4 ± 0.3) nmol NADH/mg protein/min. Liver ADH activity is somewhat blunted in mice fed MDHFD containing ethanol as compared to mice fed HFD containing ethanol although this change is not significant.
**CYP2E1 activity in the liver**

As reported previously (116), cytochrome P450 2E1 (CYP2E1) activity was increased in the liver by ethanol. Here, chronic alcohol administration significantly increased CYP2E1 activity in mice fed HFD containing ethanol (2.6 ± 0.3 nmol 4-nitrocatechol/mg protein/min) compared to HFD control (0.48 ± 0.06 nmol 4-nitrocatechol/mg protein/min) and all other groups (Fig. 4). CYP2E1 activity was also significantly increased in mice fed MDHFD containing ethanol (1.4 ± 0.2 nmol 4-nitrocatechol/mg protein/min) as compared to MDHFD control (0.52 ± 0.03 nmol 4-nitrocatechol/mg protein/min). Notably, there was a significant reduction, nearly 50%, of CYP2E1 activity in mice fed MDHFD containing ethanol as compared to those fed HFD containing ethanol.

**Aminothiols in liver**

There was no significant difference in reduced glutathione (GSH) within the enterally treated groups, although it appears that levels in mice fed HFD containing ethanol are slightly reduced compared to HFD control and MDHFD containing ethanol (Table 3). There was a significant increase in oxidized glutathione (GSSG) in mice fed HFD containing ethanol (0.68 ± 0.09 nmol / mg protein) as compared to mice fed HFD control (0.41 ± 0.07 nmol / mg protein), MDHFD control (0.33 ± 0.07 nmol / mg protein) and MDHFD containing ethanol (0.22 ± 0.01 nmol / mg protein). The GSH:GSSG is often used as an indicator of oxidative stress. Here, we found that the GSH:GSSG ratio in mice fed MDHFD control (229 ± 77 nmol / mg protein) and MDHFD containing ethanol (268 ± 21 nmol / mg protein) was greatly increased compared to mice fed HFD containing ethanol (84 ± 19 nmol / mg protein). We observed no significant differences in cysteine
levels across all groups. Cysteinylglycine (Cys-Gly), a prooxidant, was significantly elevated in mice fed HFD containing ethanol (1.24 ± 0.08 nmol / mg protein) compared to mice fed MDHFD containing ethanol (0.41 ± 0.03 nmol / mg protein) and naïve animals (0.43 ± 0.06 nmol / mg protein). Levels of glutaminecysteine (Glu-Cys) were significantly reduced in all enterally treated animals compared to naïve animals (0.33 ± 0.03 nmol / mg protein) (Table 3). We observed significant increases of SAM, the SAM:SAH ratio, and SAH concentrations in mice fed MDHFD containing ethanol as compared to mice fed HFD containing ethanol (Figure 5). We observed no significant difference in homocysteine levels between groups.
CHAPTER 5

Discussion

Taken together, the results demonstrate that dietary methyl donor supplementation (methionine, choline, betaine, folate, vitamin B12, and zinc) prevented alcohol-induced liver injury. Methyl donor supplementation also significantly increased hepatic GSH:GSSG ratios, reduced hepatic Cys-Gly concentrations, reduced liver:body weight ratios, reduced cell proliferation, blunted urine alcohol cycling and favored catalase-dependent alcohol metabolism in the liver (Scheme 1). It is also important to note that our regimen of methyl donors significantly increased hepatic concentrations of SAM by 22 fold, SAH by 3 fold, and the SAM:SAH ratio by 7 fold. Chronic alcohol exposure has been linked with deficiencies in SAM, folate, zinc, and endogenous betaine (62, 100, 117, 118). The mitigation of disease severity in animal models of ALI has been achieved through dietary supplementation of individual methionine metabolites including SAM, betaine and zinc. (72, 90, 119).

Our model is the first to show that a full regimen of methyl donors has the ability to attenuate alcohol-induced liver injury. In mice, SAM treatment has been shown to decrease steatosis and necrosis as well serum ALT activity (60). In these studies, SAM supplementation increased the hepatic SAM pool which in turn increased synthesis of GSH. We have shown that a regimen of methyl donors has the ability to significantly increase hepatic SAM even in the presence of ethanol (Fig. 5); however, we observed no significant increase in GSH concentration. SAM and betaine supplementation has been shown to attenuate fatty liver (90) and we observed a significant reduction in steatosis with our methyl donor regimen. Moreover, SAM and betaine supplementation have been shown to attenuate inflammation (60, 90); however we did not observe a reduction of
inflammation with methyl donor supplementation. SAM and betaine supplementation have been shown to attenuate necrosis (90) and it is important to note that our methyl donor supplementation completely blocked necrosis.

Chronic ethanol feeding has been shown to increase hepatic concentrations of homocysteine and SAH (90, 120). Interestingly, betaine supplementation during chronic alcohol administrations has also been shown to lower homocysteine and increase SAH concentrations (90, 121). We observed no decrease in hepatic homocysteine concentrations with methyl donor supplementation but we did in fact see increased concentrations of SAH. SAM is converted to SAH by transferring the methyl group to diverse biological acceptors (44), therefore the fact that we observed increased SAM concentrations with mice fed MDHFD containing ethanol may account for elevated levels of SAH in these same animals. SAH is then converted to homocysteine and adenosine in a reversible reaction catalyzed by SAH hydrolase (44). We did not observe significant increases in hepatic homocysteine or adenosine (data not shown) with methyl donor supplementation; therefore, another possible explanation for increased SAH concentrations may be due to alterations of SAH hydrolase by the combination of methyl donors and ethanol.

Reduced glutathione (GSH) plays a critical role in the cellular detoxification processes including the metabolism of peroxides, the conjugation with electrophiles and the scavenging of the free radicals. The relevance of glutathione in ethanol-induced liver injury is the role it plays in compensating for alcohol-related oxidative stress. Oxidative stress is a disturbance in the pro-oxidant/antioxidant balance (increased pro-oxidant levels), which may be achieved by an enhancement of oxidative reactions leading to the increased formation of reactive oxygen species (ROS) and/or by a decrease of cellular...
antioxidant defenses. Elevated glutathione levels not only protect hepatocytes from oxidative stress, but they also have been shown to rescue hepatocytes from necrosis and inflammation caused by TNF-α toxicity (58).

Research has shown that ethanol itself, not its metabolites, causes depletion of liver GSH (122). We have shown that UAC levels are much higher in the mice fed HFD containing ethanol; therefore, the direct insult by ethanol on GSH could be causing the moderate, non-significant decreases in GSH in this particular group. Researchers have shown that increased hepatic SAM concentrations leads to increased GSH production. We did not observe increased GSH concentrations with methyl donor supplementation, although we can say that methyl donors maintained GSH of ethanol treated animals comparable to control levels. A similar trend was observed in a study involving L-2-oxothiazolidine-4-carboxylic acid (OTC), a cysteine prodrug that maintains glutathione in tissues. Researchers showed that OTC blocked ethanol-induced elevations in serum aspartate transaminase (AST), necrosis and inflammation but not steatosis in rats (123). In these animals, hepatic GSH levels were not affected by ethanol or OTC treatment. Interestingly, OTC increased circulating plasma glutathione (GSH) levels about 2-fold. Additionally, superoxide production was higher in Kupffer cells isolated from ethanol-treated rats, an effect blunted by OTC.

GSH:GSSG balance is crucial to homeostasis, stabilizing the cellular biomolecular spectrum, and facilitating cellular performance and survival. The GSH:GSSG ratio is often times used as an indicator of oxidative stress and can be useful to monitor the effectiveness of antioxidant intervention strategies. We saw significant reduction of GSSG concentration with methyl donor supplementation which subsequently increased GSH:GSSG ratios in mice fed MDHFD. Cysteinylglycine (Cys-
Gly), a prooxidant generated during catabolism of glutathione, has been suggested to induce oxidative damage on DNA bases and lipid peroxidation (124, 125). A case-control study reported a significantly positive association between serum Cys-Gly levels and risk for ischemic heart disease (126). Increased amounts of ethanol in mice fed HFD containing ethanol could also be inducing the catabolism of GSH which might explain the observed increases in Cys-Gly in this group. The specific deleterious effects of increased Cys-Gly are unknown.

Mice fed HFD containing ethanol had typical UAC cycle patterns (30 to 550 mg/dl); whereas UAC cycles in the mice fed MDHFD containing ethanol was severely blunted (10 to 350 mg/dl) (Fig.1A). Moreover, mean daily UAC showed the same pattern where mice fed MDHFD containing ethanol was significantly lower, 123 ± 17 mg/dl, than mice fed HFD containing ethanol, 225 ± 34 mg/dl, even though both groups were administered diets with analogous ethanol concentrations. Importantly, we saw no differences in BAC and LAC between the two ethanol groups at sacrifice; therefore, we believe that the same dose of alcohol was reaching the liver. The UAC cyclic phenomenon may have some relevance to the pathogenesis of ALD. It has been shown that serum ALT was higher at the troughs of the UAC cycle compared with the ALT levels when UAC peaked, indicating that the liver injury may be occurring at a time when \( O_2 \) consumption is increased (43). Research has shown that increasing thyroid hormone in diet can eliminate the UAC cycle and fatty liver caused by ethanol, but unfortunately the increase in the metabolic rate leads to centrilobular necrosis and scarring of the liver in rats fed ethanol because of the centrilobular hypoxia that results from the increase in the rate of \( O_2 \) consumption by the periportal hepatocytes (43). Here we have shown that
methyl donor supplementation severely blunts the UAC cyclic pattern and therefore may be responsible for attenuation of liver injury.

Body temperature cycles inversely to UAC suggesting that the rate of metabolism could be mechanistically involved in the rate of ethanol elimination during the cycle (43). A correlation has been shown between blood levels of alcohol and induction of CYP2E1 as alcohol cycles in the enteral model of continuous alcohol administration. Notably, the level of CYP2E1 correlates with the degree of pathology (35). We observed a similar trend where increased CYP2E1 activity was correlated with more severe liver injury. Betaine feeding alone did not abolish the induction of CYP2E1 by ethanol (90). However, we observed a significant reduction, nearly 50%, of CYP2E1 activity in mice fed MDHFD containing ethanol as compared to those fed HFD containing ethanol. This trend does not support the idea that CYP2E1 activity is involved in increased ethanol metabolism in the presence of methyl donors because if CYP2E1 activity was responsible for increased ethanol metabolism in the liver, then we should have seen higher CYP2E1 activity in animals fed MDHFD containing ethanol.

A similar trend was seen in a study by Zhou et al, where zinc supplementation inhibited the activation of the CYP2E1 pathway and at the same time enhanced the ADH pathway, thus leading to the prevention of the ethanol-induced metabolic shift that is in favor of ROS production (127). On the contrary, we did not see enhanced ADH activity. In addition, researchers have shown that an acute dose of ethanol with concomitant supplementation of SAM results in a 30% inhibition of alcohol dehydrogenase ethanol metabolism. We did, however, observe a shift to catalase-dependent alcohol metabolism. We found that acyl CoA oxidase, which provides hydrogen peroxide needed for catalase-dependent alcohol metabolism, along with catalase activity were elevated in mice.
administered a MDHFD containing ethanol. This shift to catalase dependent alcohol metabolism may be supported by the fact that acyl CoA oxidase was decreased by ethanol in CYP2E1 wildtype mice but remained elevated in CYP2E1 knockout mice fed ethanol (36).

Using the intragastric-fed mouse model, we have demonstrated that methyl donor supplementation prevented alcoholic-induced decreases in SAM and GSH and alcoholic liver injury. The hepatoprotective effect of the methyl donors most likely involves in blunting of UAC cycling and the stimulation of catalase-dependent alcohol metabolism, which may account for the observable differences in urine alcohol concentrations. Moreover, methyl donor supplementation created a hepatic environment that reduced accumulation of harmful GSSG and Cys-Gly concentrations and increased hepatic detoxification capabilities. We conclude that our methyl donor regimen has the ability to optimally restore methionine-folate metabolism by increasing bioavailability of nutrients and co-factors in both the methionine cycle and the folate cycle. These results suggest that a regimen of methyl donors and cofactors may have potential in the prevention and treatment of alcoholic liver disease.
CHAPTER 6
Future Directions

In our study we demonstrated that methyl donor enrichment stimulated catalase-dependent alcohol metabolism. Moreover, we believe that this increased catalase and acyl CoA oxidase activity may have been responsible for increased ethanol metabolism which could help explain the reduced UAC concentrations observed in mice fed MDHFD containing ethanol. In order to prove its biological relevance, we plan to inhibit catalase activity by administering aminotriazole (AT), an irreversible inhibitor of catalase (128). We plan to follow the same experimental protocol as described in the Materials and Methods section; however, we plan to have four new diet groups; HFD containing AT, HFD containing ethanol and AT, MDHFD containing AT, and MDHFD containing ethanol and AT. We plan to add AT directly into the high fat liquid diet which will be administered through a feeding tube; therefore, we will be able to accurately control the dose of AT.

If we observe no difference in UAC between the two ethanol treated groups, meaning that UAC is not reduced with methyl donors, then we can conclude that catalase may be playing a role in increased alcohol metabolism. If we see no differences in UAC between ethanol treated groups in combination with the reappearance of liver damage in mice fed MDHFD containing ethanol and AT, then we can conclude that catalase may play a role in attenuating ALI by altering ethanol metabolism pathways and/or increasing ethanol metabolism in the liver. If we see the same trends in the proposed AT study as we observed in the previous study (see Results section) then we might conclude that
methyl donors stimulate catalase-dependent alcohol metabolism pathways but this stimulation has no relevant biological significance.

As mentioned before in the Results section, we observed reduced UAC in mice fed MDHFD containing ethanol as compared to mice fed HFD containing ethanol. However, we observed similar BAC and LAC between these two ethanol treated groups at sacrifice. Therefore, it may also be important to design a study which can compare rates of ethanol metabolism in mice fed HFD containing ethanol versus mice fed MDHFD containing ethanol. We plan to follow the exact experimental protocol as described in the Materials and Methods section; however, on day 10 of the study we plan to stop administration of liquid diet and take blood samples every 30 minutes for three hours. BAC will be tested at each time point in order to elucidate the rates of alcohol metabolism between mice fed HFD containing ethanol and mice fed MDHFD containing ethanol. If we observe the mice fed MDHFD have the ability to metabolize alcohol faster then it would support our previous findings that UAC can be reduced with methyl donor supplementation. Moreover, if mice fed MDHFD containing ethanol have the ability to metabolize ethanol faster then it may point to the mechanism of protective action by methyl donors.

Another important finding in our study was that we observed increased levels of acetaldehyde and acetate in mice fed HFD containing ethanol as compared to mice fed MDHFD containing ethanol. For this reason, methyl donors could be affecting the metabolism of acetaldehyde into acetate. Therefore, aldehyde dehydrogenase (ALDH), which is responsible for converting acetaldehyde into acetate, activity is another important measurement that we need to complete. Some researchers have suggested that acetaldehyde may be a source of liver damage because it has the ability to binds to
proteins, resulting in structural and functional alterations. If methyl donor supplementation increases ALDH activity then this could also be an important factor in the mechanism of protective action of methyl donors during chronic alcohol administration.

This study did not provide us with conclusive information to make new recommendations in the way the methyl donors should be used in as a therapy for alcoholic-induced liver injury; however, this work allowed us to take a closer look at how methyl donors can prevent alcohol-induced liver injury. In any case, our future efforts need to concentrate on the protective mechanisms of methyl donor supplementation. If we can reveal this mechanism, then we may be able to develop more efficient therapies for patients suffering from alcohol-induced hepatitis and cirrhosis. Moreover, other liver diseases like hepatitis B & C and aflatoxin B1 poisoning show similar pathological changes in the liver as ALD; therefore, new, improved therapies may also be used for patients with non-alcohol-induced liver disease.
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Appendix

Table 1. Composition of methyl donors and cofactors in IsoPro 3000 rodent chow, 3MS,ZM methyl enriched custom rodent chow and Tsukamoto-French high-fat liquid diet before (HFD) and after supplement addition (MDHFD). Values given are amount per kg.

<table>
<thead>
<tr>
<th></th>
<th>IsoPro 3000</th>
<th>3MS, ZM</th>
<th>HFD</th>
<th>MDHFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betaine</td>
<td>0</td>
<td>15 g</td>
<td>0</td>
<td>15 g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.6 g</td>
<td>15 g</td>
<td>1.8 g</td>
<td>15 g</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>1.2 mg</td>
<td>15 mg</td>
<td>1.4 mg</td>
<td>15 mg</td>
</tr>
<tr>
<td>L-methionine</td>
<td>0.49 mg</td>
<td>7.5 g</td>
<td>0</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.075 mg</td>
<td>0.15 mg</td>
<td>0.02 mg</td>
<td>0.15 mg</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>120 mg</td>
<td>660 mg</td>
<td>12 mg</td>
<td>660 mg</td>
</tr>
</tbody>
</table>

Methyl donor and cofactor composition in standard pellet diet (IsoPro 3000), methyl enriched pellet diet (3MS, ZM), high fat diet (HFD), and methyl donor enriched high fat diet (MDHFD). Values represent amount per kg.
Table 2. Effect of methyl-donor enriched diet on routine clinical parameters.

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>control</th>
<th>ethanol</th>
<th>Naive</th>
<th>control</th>
<th>ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statoasis score</td>
<td>0</td>
<td>0</td>
<td>2.8 ± 0.3</td>
<td>0</td>
<td>0.9 ± 0.4^t</td>
<td></td>
</tr>
<tr>
<td>Inflammation score</td>
<td>0.8 ± 0.2</td>
<td>1.7 ± 0.3^t</td>
<td>1.1 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Necrosis score</td>
<td>0</td>
<td>0.7 ± 0.4</td>
<td>1.7 ± 0.3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total pathology score</td>
<td>0.8 ± 0.2</td>
<td>2.3 ± 0.6^t</td>
<td>5.6 ± 0.5^c</td>
<td>1.5 ± 0.3</td>
<td>2.1 ± 0.4^t</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>33 ± 6</td>
<td>16 ± 4</td>
<td>76 ± 26^b,d</td>
<td>14 ± 2</td>
<td>28 ± 7</td>
<td></td>
</tr>
<tr>
<td>BAC (mg/dl)</td>
<td>6 ± 1</td>
<td>24 ± 5</td>
<td>194 ± 61^b,d</td>
<td>30 ± 5</td>
<td>239 ± 36^b,d</td>
<td></td>
</tr>
<tr>
<td>LAC (µg EtOH/µg protein)</td>
<td>--</td>
<td>11 ± 1</td>
<td>22 ± 5^d</td>
<td>8 ± 1</td>
<td>17 ± 2</td>
<td></td>
</tr>
<tr>
<td>UAC sacrifice (mg/dl)</td>
<td>--</td>
<td>23 ± 9</td>
<td>338 ± 102^b,d</td>
<td>20 ± 8</td>
<td>175 ± 44</td>
<td></td>
</tr>
<tr>
<td>Mean Daily UAC (mg/dl)</td>
<td>--</td>
<td>23 ± 2</td>
<td>225 ± 34^b,d,e</td>
<td>16 ± 1</td>
<td>123 ± 17^b,d</td>
<td></td>
</tr>
<tr>
<td>% Liver: Body Weight</td>
<td>5.1 ± 0.2</td>
<td>6.8 ± 0.5^t</td>
<td>8.6 ± 0.4^b,d,e</td>
<td>6.1 ± 0.1</td>
<td>6.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Proliferation (% BrdU)</td>
<td>--</td>
<td>0.1 ± 0.2</td>
<td>10.9 ± 5.4^b</td>
<td>3.6 ± 2.4</td>
<td>3.8 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE (n = 5 – 8). Abbreviations: alanine aminotransferase (ALT), blood alcohol concentration (BAC), liver alcohol concentration (LAC), urine alcohol concentration (UAC). Statistical difference (P < 0.05) in means for histopathology determined using Mann-Whitney rank sum test; ^t compared to HFD containing ethanol, ^c compared to naïve, ^d compared to all other groups. Statistical difference (P < 0.05) in means for clinical measurements determined using one-way ANOVA. ^a compared to naïve mice, ^b compared to mice fed control HFD, ^c compared to mice fed HFD containing ethanol, ^d compared to mice fed control MDHFD, ^e compared to mice fed MDHFD containing ethanol.
Table 3. Effect of methyl-donor enriched diet on liver redox state following alcohol exposure.

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>HFD control</th>
<th>HFD ethanol</th>
<th>MDHFD control</th>
<th>MDHFD ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>53 ± 3</td>
<td>63 ± 10</td>
<td>47 ± 4</td>
<td>58 ± 11</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>GSSG</td>
<td>0.48 ± 0.02</td>
<td>0.41 ± 0.07</td>
<td>0.68 ± 0.09&lt;sup&gt;b,d,e&lt;/sup&gt;</td>
<td>0.33 ± 0.07</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>113 ± 10</td>
<td>161 ± 24</td>
<td>84 ± 19</td>
<td>229 ± 77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>268 ± 21&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cysteine</td>
<td>9.5 ± 0.4</td>
<td>10.9 ± 0.6</td>
<td>10.6 ± 1.1</td>
<td>8.3 ± 0.6</td>
<td>11.0 ± 1.6</td>
</tr>
<tr>
<td>Cys-Gly</td>
<td>0.43 ± 0.06</td>
<td>0.99 ± 0.15</td>
<td>1.24 ± 0.08&lt;sup&gt;a,e&lt;/sup&gt;</td>
<td>0.71 ± 0.30</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Glu-Cys</td>
<td>0.33 ± 0.03&lt;sup&gt;b,c,d,e&lt;/sup&gt;</td>
<td>0.15 ± 0.03</td>
<td>0.20 ± 0.02</td>
<td>0.15 ± 0.03</td>
<td>0.20 ± 0.05</td>
</tr>
</tbody>
</table>

Values are mean nmol/mg protein ± SE (n = 5 – 8). Abbreviations: glutathione [reduced] (GSH), glutathione [oxidized] (GSSG), cysteinylglycine (Cys-Gly), and glutaminecysteine (Glu-Cys). Statistical difference (P < 0.05, one-way ANOVA) in means. <sup>a</sup>compared to naive mice, <sup>b</sup>compared to mice fed control HFD, <sup>c</sup>compared to mice fed HFD containing ethanol, <sup>d</sup>compared to mice fed control MDHFD or <sup>e</sup>compared to mice fed MDHFD containing ethanol.
Figure 1. Methyl donor supplementation reduced urine alcohol concentration. 
A. UACs of two representative mice administered ethanol intragastrically; one fed HFD containing ethanol and another fed MDHFD containing ethanol. The mouse fed HFD containing ethanol has a typical UAC cycle, whereas the cycle in the mouse fed MDHFD containing ethanol is severely blunted. Gray dashed line represents the dose of ethanol given. B. Mean daily urine alcohol concentration (UAC) was lower in ethanol-receiving mice fed MDHFD as compared to HFD despite similar dose of ethanol being administered to mice intragastrically. *P < 0.05, Statistically different from HFD + EtOH group by one-way ANOVA.
Figure 2. Methyl donor supplementation attenuated alcohol-induced liver injury. Severe steatosis and necrosis were evident in the liver of mice treated with HFD and ethanol but not MDHFD and ethanol after 4 weeks of enteral feeding. (A) HFD, (B) HFD and ethanol, (C) MDHFD, (D) MDHFD and ethanol, and (E) naïve mice. Representative photomicrographs stained with H&E, original magnification 100X. (F) Total pathological changes were scored as described in Methods. P < 0.05 by the Mann-Whitney rank sum test; a compared with mice fed HFD control and all other groups, b compared to mice fed HFD containing ethanol.
Figure 3. Reduction in urine alcohol concentration by methyl donors was not due to increased first pass metabolism. Stomach catalase activity was measured in mice fed with ethanol intra-gastrically for 4 weeks. Statistical difference aP < 0.05 as compared to naïve mice (one-way ANOVA, n=2-8).
Figure 4. Methyl donor supplementation stimulated catalase-dependent alcohol metabolism. Alcohol dehydrogenase activity and cytochrome P4502E1 (Cyp2E1) activity did not contribute to increased rates of alcohol metabolism in the liver of mice fed methyl-rich diet. Acyl CoA oxidase, which provides the substrate needed for catalase-dependent alcohol metabolism, along with liver catalase activity were elevated in mice administered a MDHFD containing ethanol. Values represent mean ± SE (n = 5-7). Statistical difference (P < 0.05) in means using one-way ANOVA. aP compared to all other groups, bP compared to MDHFD, and cP compared to HFD.
Figure 5. Methyl donor supplementation elevated SAM, SAH, and SAM:SAH ratio. Values represent mean ± SE (n = 5-7). Statistical difference in means using one-way ANOVA. ** p < 0.01, *** p < 0.001.
Scheme 1. Methyl donor supplementation stimulated catalase-dependent alcohol metabolism and repleted cellular GSH:GSSG levels which has been shown to be a protective mechanism in alcohol-induced liver injury.