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Aging and alcohol interact to alter hepatic DNA hydroxymethylation

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Abstract

Background—Aging and chronic alcohol consumption are both modifiers of DNA methylation but it is not yet known whether chronic alcohol consumption also alters DNA hydroxymethylation, a newly discovered epigenetic mark produced by oxidation of methylcytosine. Furthermore, it has not been tested whether aging and alcohol interact to modify this epigenetic phenomenon, thereby having an independent effect on gene expression.

Methods—Old (18 months) and young (4 months) male C57BL/6 mice were pair-fed either a Lieber-DeCarli liquid diet with alcohol (18% of energy) or an isocaloricLieber-DeCarli control diet for 5 weeks. Global DNA hydroxymethylation and DNA methylation were analyzed from hepatic DNA using a new LC/MS-MS method. Hepatic mRNA expression of the Tet enzymes and Cyp2e1 were measured via qRTPCR.

Results—In young mice, mild chronic alcohol exposure significantly reduced global DNA hydroxymethylation compared with control mice (0.22%±0.01% vs 0.29±0.06%, p = 0.004). Alcohol did not significantly alter hydroxymethylcytosine levels in old mice. Old mice fed the control diet showed decreased global DNA hydroxymethylation compared with young mice fed the control diet (0.24±0.02% vs 0.29±0.06%, p = 0.04). This model suggests an interaction between aging and alcohol in determining DNA hydroxymethylation ($p_{interaction}$ = 0.009). Expression of *Tet2* and *Tet3* enzymes was decreased in the old mice relative to the young (p < 0.005).

Conclusions—The observation that alcohol alters DNA hydroxymethylation indicates a new epigenetic effect of alcohol. This is the first study demonstrating the interactive effects of chronic alcohol consumption and aging on DNA hydroxymethylation.

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Keywords

DNA hydroxymethylation; alcohol; aging; liver; mouse

Introduction

Methylation of cytosine within the genome occurs by the enzymatic family of DNA methyltransferases (DNMTs), thereby forming methylcytosine (mC). Methylcytosine is present in an estimated 4-6% of the cytosine bases within a human genome, depending on cell type, and typically occurs in cytosine-guanine dinucleotides, called CpG sites, or dense repeats of CpG sites (referred to as CpG islands) (Lister and Ecker, 2009; Lister et al., 2009). The human genome contains about 30 million of these CpG dinucleotides that exist in either a methylated or unmethylated state (Cocozza et al., 2011). Through changes in the methylation status in or surrounding a gene, that gene's transcription may be increased or decreased (Attwood et al., 2002). This key role of DNA methylation has led to the extensive studying of this epigenetic mark in many facets of biological research, spanning the fields of neuroscience, cancer, early development and even behavior (Attwood et al., 2002; McGowan et al., 2009; Mill et al., 2008; Robertson, 2005).

While DNA methylation research is still a developing field, another epigenetic mark called hydroxymethylcytosine has been discovered and may hold its own important role in transcriptional regulation (Wu et al., 2011; Zhang et al., 2010). DNA hydroxymethylation has been proposed as an intermediate in the removal of methyl groups from cytosine bases (Münzel et al., 2011). The abundance of DNA hydroxymethylation is much lower than methylation, as less than 1% of cytosines are hydroxymethylated in mammalian DNA (Globisch et al., 2010b; Li and Liu, 2011). The percentage of DNA that is hydroxymethylated is quite variable between tissue types, with the central nervous system having the highest amount (Globisch et al., 2010a). In the mouse, the liver is one of the organs that has a low level of hydroxymethylation(Globisch et al., 2010b), while in humans the liver has a medium range of hydroxymethylation levels relative to other tissues (Li and Liu, 2011). Embryonic stem cells also have high levels of DNA hydroxymethylation, but these levels decrease during differentiation, suggesting that hydroxymethylcytosine may be associated with cell differentiation (Globisch et al., 2010a; Tahiliani et al., 2009b). Decreases in global hydroxymethylcytosine have been recorded in several types of cancer, linking this epigenetic mark to cellular transformation (Haffner et al., 2011; Jin et al., 2011; Kudo et al., 2012).

The most commonly regarded function of DNA hydroxymethylation is as an intermediate in the removal of the methyl group from methylcytosine, returning the cytosine to its unmodified form through the oxidation of methylcytosine to hydroxymethylcytosine(Globisch et al., 2010a; Jurkowski and Jeltsch, 2011). The TET (ten-eleven translocation) family of demethylase proteins is responsible for the enzymatic hydroxylation of methylcytosine to hydroxymethylcytosine in an iron (II), α-ketoglutarate and divalent oxygen dependent fashion. These same enzymes also catalyze the further conversion of hydroxymethylcytosine to unmodified cytosine through the intermediates

formylcytosine and carboxylcytosine(Ito et al., 2010; Tahiliani et al., 2009b). To date, three known mammalian enzymes are in the TET family (TET1, TET2 and TET3), with each enzyme apparently being expressed differently among tissue types. Interestingly, the de novo DNA methyltransferases(DNMT 3a/b) may also have the ability to convert hydroxymethylcytosine to formylcytosine and carboxylcytosine through the same oxidative steps (Chen et al., 2012a). In hepatocellular carcinoma, a decrease in global hydroxymethylcytosine in cancerous tissue is also associated with reduction of the TET1 enzyme (Liu et al., 2013), leading to the possibility that the TET enzymes play an active role in the development of some cancers.

In general, hydroxymethylation of cytosines within a gene body, gene promoter or CpG island is associated with an increase in transcription of that gene, the opposite pattern that is described for methylation of cytosines(Ficz et al., 2011; Jones et al., 1998; Stroud et al., 2011; Wu et al., 2011). It has therefore been proposed that hydroxymethylation may in itself act as a regulator of gene transcription through the alteration of binding affinities of the methyl-binding proteins. Most of the methyl-binding proteins will no longer bind to DNA once the methylcytosine has been hydroxylated, causing these proteins to dissociate from the DNA, perhaps changing the transcriptional status of that gene (Jin et al., 2010; Valinluck and Sowers, 2007; Valinluck et al., 2004).

Several studies have been published regarding the changes of DNA methylation with age, but there is little research involving whether hydroxymethylation is also affected with age (Richardson, 2003; Singhal et al., 1987; Wilson et al., 1987). It is known that aging alters hydroxymethylation in some cells in the central nervous system, but the effect of age in non-CNS tissues is yet to be established (Chen et al., 2012b; Chouliaras et al., 2013). Furthermore, the effect of alcohol on DNA hydroxymethylation has not been reported, even though alcohol is known to have a profound effect on other epigenetic phenomena including DNA methylation and histone modifications (Choi et al., 1999; Esfandiari et al., 2010; Sauer et al., 2010). We therefore investigated the effect of mild, chronic alcohol exposure on global DNA hydroxymethylation as well as alcohol's interaction with aging in determining the global DNA hydroxymethylation status using an animal model of chronic alcohol consumption and aging (Sauer et al., 2010). Changes in the oxidative pathways in the liver may also occur with aging and alcohol consumption. In an effort to quantify these changes we measured the expression of the cytochrome p450 gene Cyp2e1.

Materials and Methods

Animals and diets

This study was reviewed and approved by the Animal Care and Use Committee of the USDA Human Nutrition Research Center on Aging at Tufts University. Eighteen 18-month-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) and twenty 4-month-old mice were used in this study. All diets were obtained from Dyets (Easton/Bethlehem, PA).

The mice in each of the two age groups were randomly assigned into two groups. After one week of adaptation to the L-amino acid defined Lieber-DeCarli liquid control diet, one

group was fed an L-amino acid defined Lieber-DeCarli alcohol diet (Lieber et al., 1989; Sauer et al., 2010). Ethanol was present at a concentration of 3.1% (v/v), comprising 18% of total energy, and was gradually introduced over an adaptation period of 2 weeks. The control group of mice received isocaloric Lieber-DeCarli liquid control diets with an isocaloric amount of maltodextrin. Water was not provided because the liquid diets provided sufficient fluid.

Individually housed mice were group pair-fed to mice in the same age group to decrease variability of energy and nutrient intake within and between dietary groups. The amount of food supplied to each diet group was matched to the mean daily food consumption of the group within the same age category with the least food consumption. For example, mice in the young control group had the same volume of food given to them as mice in the young alcohol group (likewise for the old mice). Therefore, the volume of food in each age group was adjusted when necessary to ensure they consumed equal volumes of liquid daily. At 5 weeks, mice were killed and liver samples were harvested as previously described (Choi et al., 2003).

Global DNA methylation and hydroxymethylation

Apparatus—All experiments were performed on an Agilent 1100 LC Quaternary Pump with an Applied Biosystems 3200 Q Trap mass spectrometer-mass spectrometer system equipped with a turbospray ionization source. The liquid chromatograph used was an Agilent 1100 Series. A SuplexpKb 100 analytical column (25 cm x 2.1 mm) protected by a 5-um SuplexpKb 100 precolumn (2 cm x 2.1 mm) (Supelco, Bellefonte, PA) was used.

Reagents—The mobile phase for all reactions consisted of 7mM ammonium acetate pH 6.7 / HPLC grade-methanol 5% (v/v) and was prepared with HPLC-grade water (Fisher Scientific). Before use, mobile phase was filtered through a 0.2-um nylon membrane (Millipore, Billerica, MA). The stable isotope-labeled compounds [$^{15}N_3$]2'-deoxycytidine and the custom-made (methyl-d_3,ring-6-d_1)-5-methyl-2'-deoxycytidine (both from Cambridge Isotopes Laboratories, Inc., Andover, MA) were used as internal standards for 2'-deoxycytidine, 5-methyl-2'-deoxycytidine and 5-hydroxymethyl-2'-deoxycytidine residues.

DNA Extraction and Hydrolysis—Extraction of DNA was conducted through the standard phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] method with precipitation with 100% ethanol and 3M Sodium Acetate, pH 5.2 (Strauss, 1998). DNA was then re-dissolved in Tris-EDTA buffer. Quality of DNA was verified on an Agarose gel. DNA quantification and 260/280 ratios were measured on a Nanodrop 1000 (Thermo Scientific, Wilmington, DE). All DNA samples used had a 260/280 ratio equal to or greater than 1.8. DNA hydrolysis was performed as previously described (Friso et al., 2002). In brief, 1μg of genomic DNA was denatured by heating the sample at 100 °C for 3 minutes and subsequently chilled on ice. Next, 0.1 M ammonium acetate (pH 5.3) and 2 units of nuclease P1 (Sigma, St. Louis, MO) were then added to each sample. The mixture was incubated at 45 °C for 2 hours. Subsequently, 1 μL of 1 M ammonium bicarbonate (pH 7.8) (Sigma, St. Louis, MO) and 0.002 units of venom phosphodiesterase I (Sigma, St. Louis, MO) were

added to each sample. All samples were incubated for an additional 2 hours at 37 °C, then 0.5 units of alkaline phosphatase (Sigma, St. Louis, MO) was added to the mixture and incubated for another 1 hour at 37 °C took place. The stable isotopes [$^{15}N_3$]-2'-deoxycytidine and (methyl-d_3,ring-6-d_1)-5-methyl-2'-deoxycytidine were then added to the samples to reach a final concentration of 1 and 0.25 ng/μL, respectively. The total volume for each sample was 35 μL at the end of hydrolysis.

LC/MS-MS Procedure—The analytical column was equilibrated with the mobile phase at a flow rate of300 μL/min. A volume of 10 μL of hydrolyzed DNA was injected into the column that was kept at 25°C to separate the DNA bases by isocratic elution. Data was collected in multiple reaction monitoring (MRM) mode, using the mass transitions after ion fragmentation as follows: 2'-deoxycytidine at *m/z* 228.1 → 112.1, 5-methyl-2'-deoxycytidine at *m/z* 242.1 → 126.1, and 5-hydroxymethyl-2'-deoxycytidine at *m/z* 258.1 → 142.1.

Calculation of % unmodified cytosine, methylcytosine and hydroxymethylcytosine: The absolute mass of 2'-deoxycytidine, 5-methyl-2'-deoxycytidine and 5-hydroxymethyl-2'-deoxycytidine was calculated using the intensity and the known mass of the internal standards. The methylcytosine internal standard [(methyl-d_3,ring-6-d_1)-5-methyl-2'-deoxycytidine] was also used as the hydroxymethylcytosine internal standard. From the absolute mass of each component, the relative amount of 2'-deoxycytidine (umC), 5-methyl-2'-deoxycytidine (mC) and 5-hydroxymethyl-2'-deoxycytidine (hmC) was calculated as such:

$$\%hmC = \frac{(\text{mass of hmC})}{(\text{mass of mC}) + (\text{mass of umC}) + (\text{mass of hmC})} \times 100\%$$

Gene expression

Changes in expression of the *Tet* genes and *Cyp2e1* were determined by quantitative real-time PCR. An RNA extraction was performed using Trizol reagent and cDNA was synthesized using a standard reverse transcription kit (Invitrogen). Quantitative RT-PCR was conducted on the LightCycler 480 real-time PCR machine (Roche). Gene expression was determined as ΔCt, following normalization to the housekeeping gene *GAPDH* (ΔCt = $Ct_{GeneX} - CT_{GAPDH}$). Expression was normalized to the young control group and is displayed as relative fold difference.

Statistics

To determine differences in global cytosine modifications between the age and diet groups a twoway analysis of variance (ANOVA) followed by Tukey-Kramer adjustment for multiple comparisons was employed. The statistical model included age and diet information, as well as the interaction term for these two variables. Least-squares means were used to determine the degree of differences between groups. Differences in animal weights for all four groups at each week were determined through a oneway ANOVA, and were also adjusted for multiple comparisons using the Tukey-Kramer method.

The level of significance was $p<0.05$ for all analyses. Values in the text are means ± SEM. Error bars on all figures represent SEM. All statistical calculations were performed in SAS version 9.3.

Results

Animals

Both young and old mice fed the 18% alcohol diets lost weight in the first week but thereafter gained weight. Despite the weight difference, all mice tolerated the liquid diets very well. At the end of the feeding study, all young mice were of similar weight. Older mice fed alcohol weighed less than the control group at the end of the feeding period. Additionally, at the end of the study the young mice weighed less than the old mice, regardless of whether the old mice were fed the control or the alcohol diet(Sauer et al., 2010).

Global DNA hydroxymethylation

Overall, when both young and old mice were fed an alcohol-containing diet for five weeks there was a significant reduction in global hepatic DNA hydroxymethylation relative to those mice fed a control diet (0.27±0.01% vs 0.31±0.02%P=0.04) (**Figure 1A**). When stratified by age group, the effect of alcohol is different in the young mice from the old mice, resulting in a significant interaction ($P_{interaction}$=0.009) (**Figure 1B**). Young mice fed the alcohol diet had a significantly reduced global DNA hydroxymethylation compared with young mice fed the control diet (0.22%±0.01% vs 0.29±0.06%, P=0.0004) (Figure 1B). However, old mice fed the alcohol diet did not show any significant change in global DNA hydroxymethylation compared with old mice fed the control diet (0.26%±0.02% vs 0.24±0.02%, p=NS) (Figure 1B). Interestingly, old mice fed the control diet showed a decrease in global DNA hydroxymethylation relative to the with young control mice, demonstrating the effect of age alone on hydroxymethylation (0.24±0.02% vs 0.29±0.06%, P=0.04) (Figure 1B).

Global DNA methylation

Similar to hydroxymethylation, feeding alcohol to mice seemed to have a different effect on young mice than it did in old mice ($P_{interaction}$=0.05) (**Figure 2**). Old mice fed a control diet demonstrated a decrease in global DNA methylation compared with young mice fed the same diet, though these differences did not quite reach significance (4.49±0.19% vs 4.84±0.20%, P=0.09) (Figure 2). Similarly, old mice fed a diet containing alcohol tended to have reduced global DNA methylation levels relative to young mice also fed alcohol (4.33±0.26% vs 4.80±0.15%, P=0.09) (Figure 2). Chronic alcohol consumption did not significantly reduce global DNA methylation status in both young (4.84 ± 0.20% vs 4.80 ± 0.15%, P=NS) and old mice (4.49 ± 0.19% vs4.33 ± 0.26%, P=NS) (Figure 2).

Global unmodified cytosine

Overall, old mice had an increased percentage of cytosines that were unmodified than young mice (95.33±0.09% vs 94.93±0.07%, P=0.001) (**Figure 3A**). When broken down in the two diet types, the changes in the proportion of unmodified cytosine are opposite to the changes

in those of methyl cytosine. Old mice fed either the control or alcohol diet showed significantly increased unmodified cytosine levels compared to their young mouse counterpart (control: 95.27±0.19% vs 94.87±0.17%, *P*=0.05; alcohol: 95.41±0.27% vs 94.98±0.15%, *P*=0.01) (**Figure 3B**).

Expression of Tet Enzymes

Tet1 mRNA was minimally expressed in all samples, as expected from previous reports demonstrating that TET1 is mainly found in embryonic stem cells (Szwagierczak et al., 2010; Tahiliani et al., 2009a). Expression of both *Tet2* and *Tet3* mRNA was reduced in the older mice, regardless of their diet (**Figure 4A**). Old mice fed alcohol had decreased expression of the *Tet2* gene (*P*= 0.03) (**Figure 4B**), and also tended to exhibit reduced expression of *Tet3* (*P*=0.06) (**Figure 4C**) relative to the young mice fed alcohol.

Expression of Cyp2e1 Gene

The cytochrome p450 enzyme Cyp2e1 is involved in the microsomal ethanol oxidizing system and is a potent producer of oxidative species, especially that in response to alcohol (Albano, 2006; Cederbaum et al., 2009). The gene expression of *Cyp2e1*was reduced in the older mice regardless of their diet, but did not change in either the young or old mice when fed alcohol (**Figure 5**).

Discussion

Using a new LC/MS-MS method and hepatic DNA from a mouse model of chronic alcohol consumption and aging, we investigated whether alcohol consumption and aging alter hepatic DNA hydroxymethylation. Overall, we observed that alcohol consumption has a significant impact on global DNA hydroxymethylation and this effect is age-specific. To our knowledge this is the first observation that alcohol consumption changes DNA hydroxymethylation, an epigenetic phenomenon that may affect gene expression independently from DNA methylation. In this model, alcohol interacts with aging in determining the global DNA hydroxymethylation.

In our study young mice showed significantly reduced global DNA hydroxymethylation when they were fed a Lieber-DeCarli alcohol diet, while levels of methylcytosine and unmodified cytosine were not affected. Unlike in the young mice, old mice fed the alcohol diet did not show any significant changes in hydroxymethylcytosine or methylcytosine. This result suggests that hepatic DNA in the old mice might be more resistant to the effect of alcohol on the conversion of methylcytosine to hydroxymethylcytosine compared with young mice. The amount of unmodified cytosine was higher in the old mice fed alcohol relative to young mice fed alcohol; therefore we may also speculate that alcohol equally affects the conversion processes from methylcytosine to hydroxymethylcytosine and from hydroxymethylcytosine to unmodified cytosine. Even though there was no significant difference, in comparing old mice fed an alcohol diet to old mice fed a control diet, the alcohol diet fed mice demonstrated a decreased level of methylcytosine and increased levels hydroxymethylcytosine and unmodified cytosine. To understand these changes, we need to more fully understand the dynamics of modified cytosine distribution in DNA. The

insignificant decrease in methylcytosine levels and insignificant increase in unmodified cytosine levels may partially support the hypothesis that alcohol enhances the process from methylcytosine to unmodified cytosine through hydroxymethylcytosine.

Alcohol is known to decrease S-adenosylmethionine (SAM), which is the unique methyl donor to DNA methylation, and increase S-adenosylhomocysteine (SAH), an inhibitor of DNA methylation reactions (Selhub, 2002). It has been expected that chronic alcohol consumption will decrease global DNA methylation through SAM and SAH changes, however previous studies have not clearly demonstrated significant effects of alcohol on hepatic DNA methylation (Stickel et al., 2000; Trimble et al., 1993). In the present study, old mice fed either the alcohol or control diet had reduced global DNA methylation compared to young mice. In general aging is known to decrease global DNA methylation, even though this effect is tissue specific and was not witnessed in this study (Vanyushin et al., 1973). The reduction in DNA methylation in old mice fed the alcohol diet compared to young mice fed the same diet was greater than the difference in mice fed the control diet, and nearly reached significance. From this data we speculate that alcohol may enhance the effect of aging on DNA methylation, even though alcohol may not alter DNA methylation patterns on its own.

Inhibition of the TET family of enzymes that catalyze the conversion from methylcytosine to hydroxymethylcytosinemay occur through the addition of alcohol to the diet, which would potentially result in an increase in methylcytosine and a decrease in hydroxymethylcytosine, similar to what we see in the young mice. We also speculate that alcohol continues to oxidize hydroxymethylcytosine to formylcytosine and carboxylcytosine, steps in the conversion from hydroxymethylation back to unmodified cytosine (Globisch et al., 2010b). Therefore, the oxidation of hydroxymethylcytosine to formylcytosine may be occurring more rapidly than the oxidation of methylcytosine to hydroxymethylcytosine in the livers of young mice. It is important to note that since the proportion of total cytosine that is in the hydroxymethylcytosine form is much smaller than the proportions of methylcytosine and unmodified cytosine, even significant changes in hydroxymethylation may not be strong enough to induce significant changes in the overall levels of methylcytosine and unmodified cytosine.

Because it is known that the conversion of methylcytosine to hydroxymethylcytosine is an oxidative reaction, and alcohol has a strong oxidative effect in the liver, the results from this study seem opposite than expected. However, we did not find significant changes in *Cyp2e1* mRNA expression in the mice fed alcohol relative to their control diet counterparts. Because Cyp2e1 is a biomarker for oxidative stress, particularly in response to alcohol, the lack of changes in Cyp2e1 expression in response to feeding a chronic alcoholic diet suggests that the amount of alcohol in the diet, or the duration of the diet was not adequate to elicit an oxidative response (Albano, 2006; Cederbaum et al., 2009; Lieber, 1997). This model for chronic alcohol consumption is mild relative to other rodent studies, as only 18% of total calories were derived from alcohol compared to the standard Lieber-DeCarli diet containing 36% of total calories from alcohol (DeCarli and Lieber, 1967). It is possible that post translational modifications occurred that ultimately altered the amount of Cyp2e1 protein in

the cells, although studies have indicated that chronic alcohol consumption increases both mRNA and protein levels of Cyp2e1 (Novak and Woodcroft, 2000).

One interesting observation is that old mice fed the control liquid diet had significantly lower global DNA hydroxymethylation compared with young mice fed the same control diet. Previous studies demonstrated that aging increased DNA hydroxymethylation and expression of TET enzymes in the brain, though these results varied (Dzitoyeva et al., 2012; Szulwach et al., 2011). As previously mentioned, TET enzymes catalyze the conversion of methylcytosine to hydroxymethylcytosine, as well as catalyze the further conversion of hydroxymethylcytosine to unmodified cytosine. We do not know if this age-related difference in hydroxymethylation is from liver-specific changes in TET expression, or is a differential response of old mice to the control Lieber-DeCarli liquid diet compared with young mice. We have found here that the expression of both *Tet2* and *Tet3* mRNA was decreased in all old mice relative to young, which was similar to the changes we found in hydroxymethylation. Additionally, old mice exhibited less expression of *Cyp2e1* relative to young mice, regardless of the diet, which is opposite than expected from previous literature (Lieber, 2004; Wauthier et al., 2006). These decreases in *Tet2* and *Tet3* mRNA, as well as the potential decrease in an oxidative environment as demonstrated by *Cyp2e1* expression may explain in part the decrease in hydroxymethylcytosine that we see in old mice. Interestingly, there were no significant changes in global DNA hydroxymethylation between old and young mice fed the alcohol diet, indicating that aging alone alters hepatic DNA hydroxymethylation but in combination with chronic alcohol consumption there is a disruption in this aging effect.

Conclusion

The observation that alcohol alters DNA hydroxymethylation but not DNA methylation indicates a new epigenetic effect of alcohol, and indicates that hydroxymethylation may be more sensitive to the effects of chronic alcohol consumption. Gene specific DNA hydroxymethylation analysis is needed to determine the effects of alcohol on DNA hydroxymethylation at a specific gene or specific area of the DNA. This will be especially important and interesting in identifying genes associated with aging and chronic alcohol liver injury. Furthermore, since decreases in hydroxymethylation are common finding in cancer, perhaps the effect of aging and alcohol consumption provides an environment for cancer development in the liver through this epigenetic mechanism.

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Literature Cited

Albano E. Alcohol, oxidative stress and free radical damage. Proceedings of the nutrition society. 2006; 65:278–290. [PubMed: 16923312]

Attwood J, Yung R, Richardson B. DNA methylation and the regulation of gene transcription. Cellular and Molecular Life Sciences CMLS. 2002; 59:241–257. [PubMed: 11915942]

Cederbaum AI, Lu Y, Wu D. Role of oxidative stress in alcohol-induced liver injury. Archives of toxicology. 2009; 83:519–548. [PubMed: 19448996]

Chen C-C, Wang K-Y, Shen C-KJ. The mammalian de novo DNA methyltransferases DNMT3A and DNMT3B are also DNA 5-hydroxymethylcytosine dehydroxymethylases. Journal of Biological Chemistry. 2012a; 287:33116–33121. [PubMed: 22898819]

Chen H, Dzitoyeva S, Manev H. Effect of aging on 5-hydroxymethylcytosine in the mouse hippocampus. Restorative Neurology and Neuroscience. 2012b; 30:237–245. [PubMed: 22426040]

Choi SW, Friso S, Dolnikowski GG, Bagley PJ, Edmondson AN, Smith DE, Mason JB. Biochemical and molecular aberrations in the rat colon due to folate depletion are age-specific. J Nutr. 2003; 133:1206–1212. [PubMed: 12672944]

Choi SW, Stickel F, Baik HW, Kim YI, Seitz HK, Mason JB. Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. J Nutr. 1999; 129:1945–1950. [PubMed: 10539767]

Chouliaras L, Mastroeni D, Delvaux E, Grover A, Kenis G, Hof PR, Steinbusch HW, Coleman PD, Rutten BP, van den Hove DL. Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer's disease patients. Neurobiology of aging. 2013

Cocozza S, Akhtar MM, Miele G, Monticelli A. CpG Islands Undermethylation in Human Genomic Regions under Selective Pressure. PloS one. 2011; 6:e23156. [PubMed: 21829712]

DeCarli LM, Lieber CS. Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet. The Journal of nutrition. 1967; 91:331–336. [PubMed: 6021815]

Dzitoyeva S, Chen H, Manev H. Effect of aging on 5-hydroxymethylcytosine in brain mitochondria. Neurobiology of aging. 2012

Esfandiari F, Medici V, Wong DH, Jose S, Dolatshahi M, Quinlivan E, Dayal S, Lentz SR, Tsukamoto H, Zhang YH, et al. Epigenetic regulation of hepatic endoplasmic reticulum stress pathways in the ethanol-fed cystathionine beta synthase-deficient mouse. Hepatology. 2010; 51:932–941. [PubMed: 19957376]

Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature. 2011; 473:398–402. [PubMed: 21460836]

Friso S, Choi S-W, Dolnikowski GG, Selhub J. A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass spectrometry. Analytical chemistry. 2002; 74:4526–4531. [PubMed: 12236365]

Globisch D, Munzel M, Muller M, Michalakis S, Wagner M, Koch S, Bruckl T, Biel M, Carell T. Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One. 2010a; 5:e15367. [PubMed: 21203455]

Globisch D, Münzel M, Müller M, Michalakis S, Wagner M, Koch S, Brückl T, Biel M, Carell T. Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PloS one. 2010b; 5:e15367. [PubMed: 21203455]

Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget. 2011; 2:627. [PubMed: 21896958]

Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature. 2010

Jin S-G, Jiang Y, Qiu R, Rauch TA, Wang Y, Schackert G, Krex D, Lu Q, Pfeifer GP. 5-Hydroxymethylcytosine is strongly depleted in human cancers but its levels do not correlate with IDH1 mutations. Cancer research. 2011; 71:7360–7365. [PubMed: 22052461]

Jin SG, Kadam S, Pfeifer GP. Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic acids research. 2010; 38:e125. [PubMed: 20371518]

Jones PL, Veenstra GCJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nature genetics. 1998; 19:187–191. [PubMed: 9620779]

Jurkowski TP, Jeltsch A. Burning off DNA Methylation: New Evidence for Oxygen-Dependent DNA Demethylation. ChemBioChem. 2011

Kudo Y, Tateishi K, Yamamoto K, Yamamoto S, Asaoka Y, Ijichi H, Nagae G, Yoshida H, Aburatani H, Koike K. Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation. Cancer science. 2012; 103:670–676. [PubMed: 22320381]

Li W, Liu M. Distribution of 5-hydroxymethylcytosine in different human tissues. Journal of nucleic acids 2011. 2011

Lieber CS. Cytochrome P-4502E1: its physiological and pathological role. Physiological reviews. 1997; 77:517–544. [PubMed: 9114822]

Lieber CS. Cyp2e1: From ash to nash. Hepatology research. 2004; 28:1–11. [PubMed: 14734144]

Lieber CS, DeCarli LM, Sorrell MF. Experimental methods of ethanol administration. Hepatology. 1989; 10:501–510. [PubMed: 2673971]

Lister R, Ecker JR. Finding the fifth base: genome-wide sequencing of cytosine methylation. Genome research. 2009; 19:959–966. [PubMed: 19273618]

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. 2009; 462:315–322. [PubMed: 19829295]

Liu C, Liu L, Chen X, Shen J, Shan J, Xu Y, Yang Z, Wu L, Xia F, Bie P. Decrease of 5-Hydroxymethylcytosine Is Associated with Progression of Hepatocellular Carcinoma through Downregulation of TET1. PloS one. 2013; 8:e62828. [PubMed: 23671639]

McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonté B, Szyf M, Turecki G, Meaney MJ. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nature neuroscience. 2009; 12:342–348.

Mill J, Tang T, Kaminsky Z, Khare T, Yazdanpanah S, Bouchard L, Jia P, Assadzadeh A, Flanagan J, Schumacher A. Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. The American Journal of Human Genetics. 2008; 82:696–711.

Münzel M, Globisch D, Carell T. 5-Hydroxymethylcytosine, the Sixth Base of the Genome. Angewandte Chemie International Edition. 2011; 50:6460–6468.

Novak RF, Woodcroft KJ. The alcohol-inducible form of cytochrome P450 (CYP2E1): role in toxicology and regulation of expression. Archives of pharmacal research. 2000; 23:267–282. [PubMed: 10976571]

Richardson B. Impact of aging on DNA methylation. Ageing research reviews. 2003; 2:245–261. [PubMed: 12726774]

Robertson KD. DNA methylation and human disease. Nature Reviews Genetics. 2005; 6:597–610.

Sauer J, Jang H, Zimmerly EM, Kim KC, Liu Z, Chanson A, Smith DE, Mason JB, Friso S, Choi SW. Ageing, chronic alcohol consumption and folate are determinants of genomic DNA methylation, p16 promoter methylation and the expression of p16 in the mouse colon. Br J Nutr. 2010:1–7.

Selhub J. Folate, vitamin B12 and vitamin B6 and one carbon metabolism. The journal of nutrition, health & aging. 2002; 6:39–42.

Singhal RP, Mays-Hoopes LL, Eichhorn GL. DNA methylation in aging of mice. Mechanisms of ageing and development. 1987; 41:199–210. [PubMed: 3431172]

Stickel F, Choi SW, Kim YI, Bagley PJ, Seitz HK, Russell RM, Selhub J, Mason JB. Effect of chronic alcohol consumption on total plasma homocysteine level in rats. Alcohol Clin Exp Res. 2000; 24:259–264. [PubMed: 10776661]

Strauss WM. Preparation of genomic DNA from mammalian tissue. Current protocols in molecular biology. 1998:2.2.1–2.2.3.

Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE. 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol. 2011; 12:R54. [PubMed: 21689397]

Szulwach KE, Li X, Li Y, Song C-X, Wu H, Dai Q, Irier H, Upadhyay AK, Gearing M, Levey AI. 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. Nature neuroscience. 2011; 14:1607–1616.

Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H. Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. Nucleic Acids Research. 2010; 38:e181–e181. [PubMed: 20685817]

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009a; 324:930–935. [PubMed: 19372391]

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009b; 324:930–935. [PubMed: 19372391]

Trimble KC, Molloy AM, Scot JM, Weir DG. The effect of ethanol on one-carbon metabolism: Increased methionine catabolism and lipotrope methyl-group wastage. Hepatology. 1993; 18:984–989. [PubMed: 7691709]

Valinluck V, Sowers LC. Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer research. 2007; 67:946–950. [PubMed: 17283125]

Valinluck V, Tsai H-H, Rogstad DK, Burdzy A, Bird A, Sowers LC. Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). Nucleic Acids Research. 2004; 32:4100–4108. [PubMed: 15302911]

Vanyushin BF, Mazin AL, Vasilyev VK, Belozersky AN. The content of 5-methylcytosine in animal DNA: the species and tissue specificity. Biochim Biophys Acta. 1973; 299:397–403. [PubMed: 4735514]

Wauthier V, Schenten V, Verbeeck RK, Calderon PB. Ageing is associated with increased expression but decreased activity of CYP2E1 in male Wistar rats. Life sciences. 2006; 79:1913–1920. [PubMed: 16904701]

Wilson VL, Smith R, Ma S, Cutler R. Genomic 5-methyldeoxycytidine decreases with age. Journal of Biological Chemistry. 1987; 262:9948–9951. [PubMed: 3611071]

Wu H, D'Alessio AC, Ito S, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y. Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes & development. 2011; 25:679–684. [PubMed: 21460036]

Zhang H, Zhang X, Clark E, Mulcahey M, Huang S, Shi YG. TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. Cell research. 2010; 20:1390–1393. [PubMed: 21079648]

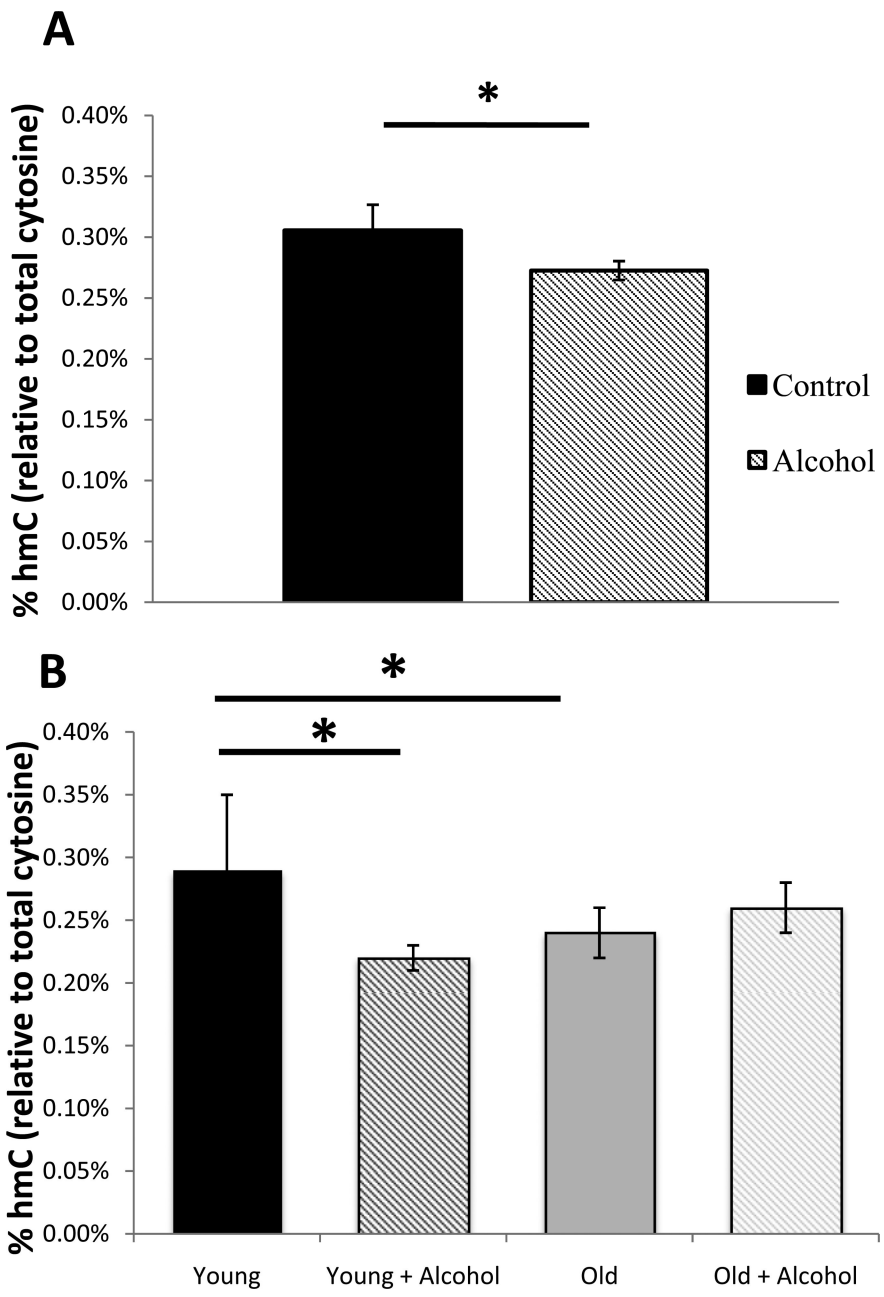


Figure 1.

(A) Percentage of cytosine that is hydroxymethylated in the hepatic DNA of mice fed either a control or alcohol Lieber-DeCarli diet. Regardless of age, mice fed alcohol had reduced hepatic hydroxymethylcytosine. (B) Percentage of cytosine that is hydroxymethylated in the hepatic DNA of young or old mice fed either a control or alcohol Lieber-DeCarli diet. Alcohol in the young, but not old, mice reduces hepatic hydroxymethylcytosine. Aging without alcohol consumption is also associated with a decrease in hydroxymethylcytosine. Aging and alcohol interact to modify hydroxymethylation ($P_{interaction}$=0.009). * Signifies a *P*-value < 0.05

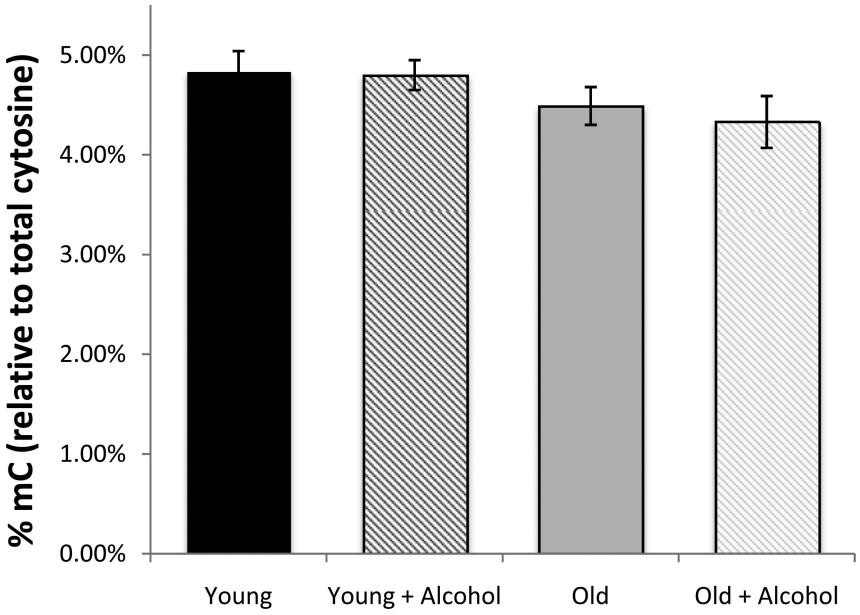


Figure 2.

Percentage of cytosine that is methylated in the hepatic DNA of young or old mice fed either a control or alcohol Lieber-DeCarli diet. Aging and alcohol interact to modify methylation ($P_{interaction}$=0.05).

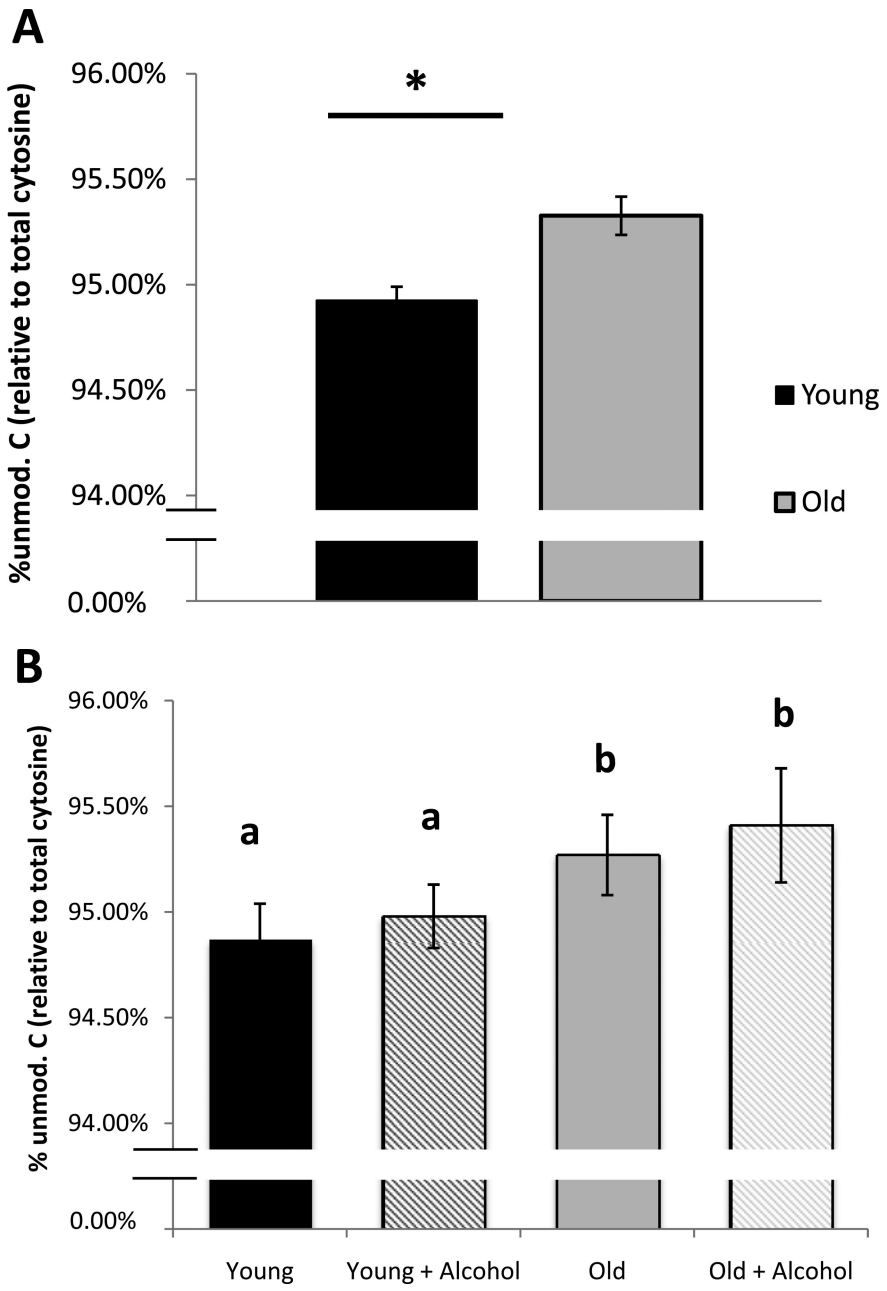
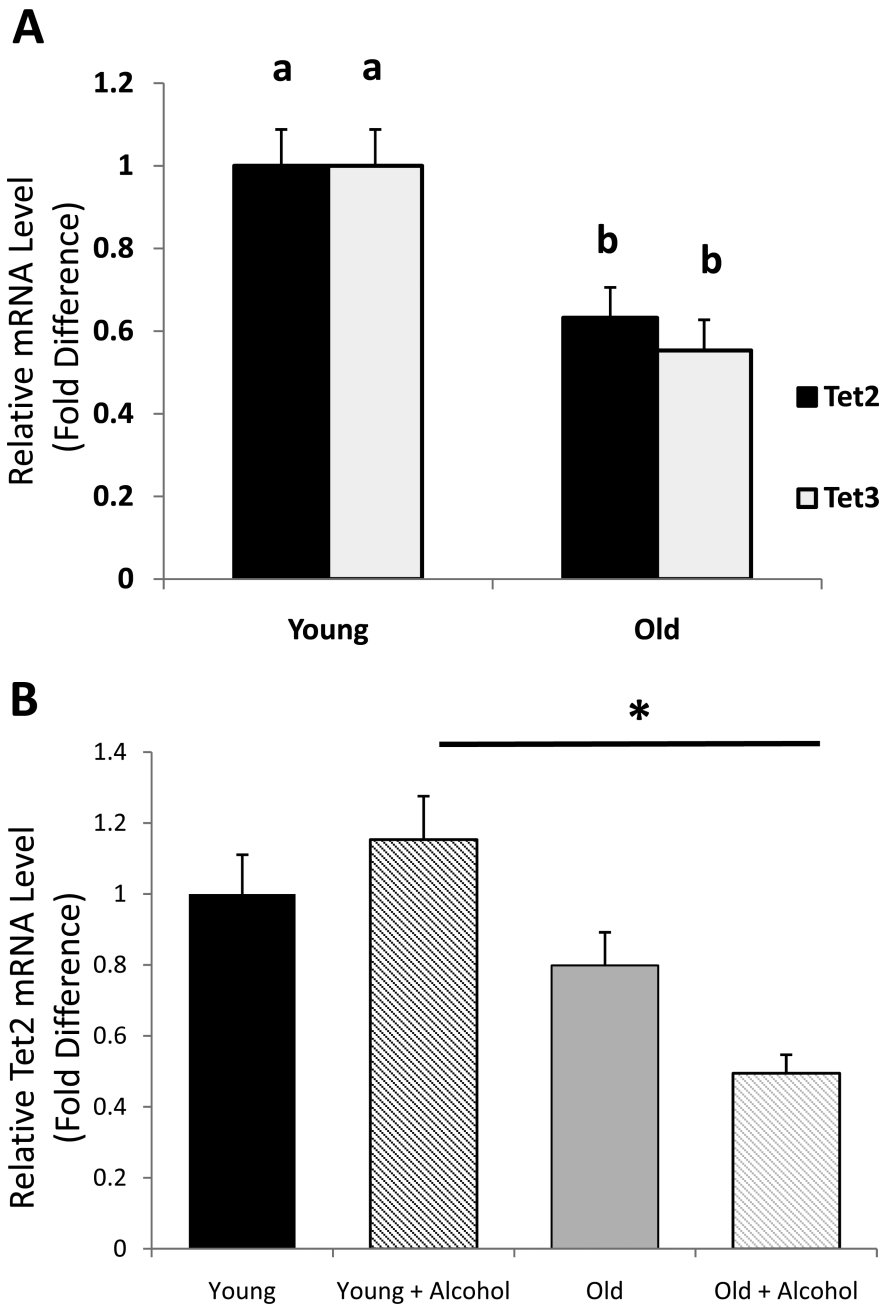


Figure 3.

(A) Percentage of cytosine that is unmodified in the hepatic DNA of young and old mice, regardless of the diet. More cytosine exists in the unmodified form in the old mice. * Signifies a *P*-value < 0.05. (B) Percentage of cytosine that is unmodified in the hepatic DNA of young or old mice fed either a control or alcohol Lieber-DeCarli diet. Different letters represent significant differences (*P*-value < 0.05).



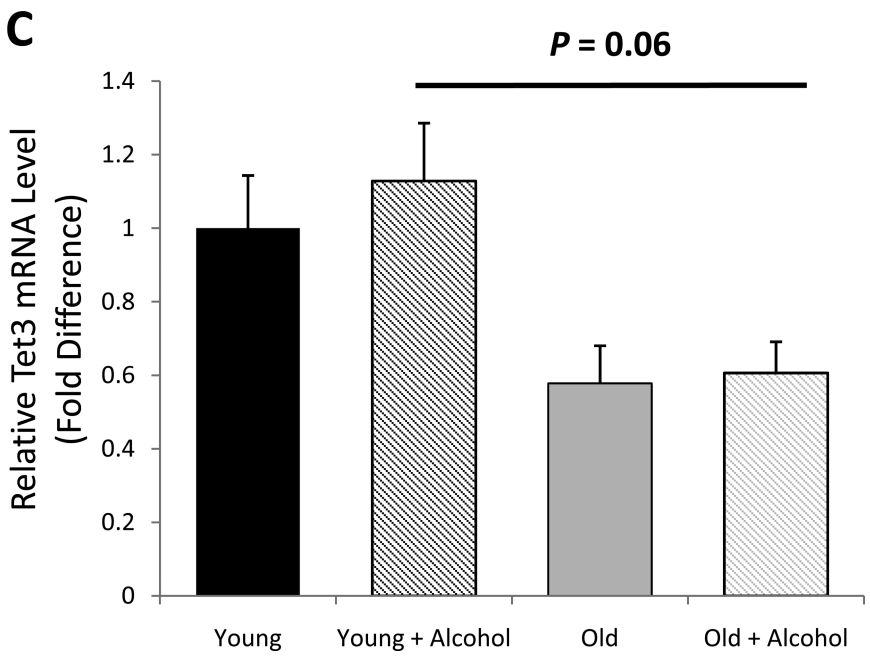


Figure 4.

(A) Expression of the *Tet2* and *Tet3* genes, the DNA demethylase enzymes, relative to the young mice regardless of diet. Different letters represent significant differences (*P*-value < 0.05). (B) Expression of *Tet2* in young and old mice fed either a control or alcohol Lieber-DeCarli diet. Alcohol significantly reduced expression in the old mice relative to the young. * Signifies a *P*-value < 0.05 (C) Expression of *Tet3* in young and old mice fed either a control or alcohol Lieber-DeCarli diet. Alcohol tended to reduced expression in the old mice relative to the young.

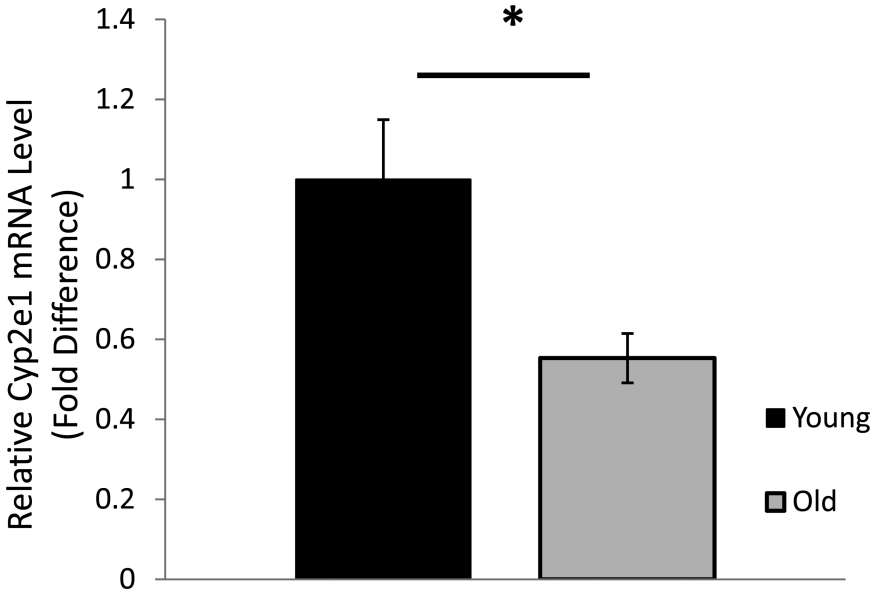


Figure 5.

Expression of *Cyp2e1*, a marker of oxidative stress, relative to the young mice. * Signifies a *P*-value < 0.05