Iron Supplementation Reverses the Reduction of Hydroxymethylcytosine in Hepatic DNA Associated With Chronic Alcohol Consumption in Rats

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Background: Alcohol is known to affect two epigenetic phenomena, DNA methylation and DNA hydroxymethylation, and iron is a cofactor of ten-eleven translocation (TET) enzymes that catalyze the conversion from methylcytosine to hydroxymethylcytosine. In the present study we aimed to determine the effects of alcohol on DNA hydroxymethylation and further effects of iron on alcohol associated epigenetic changes.

Methods: Twenty-four male Sprague-Dawley rats were fed either Lieber-DeCarli alcohol diet (36% calories from ethanol) or Lieber-DeCarli control diet along with or without iron supplementation (0.6% carbonyl iron) for 8 weeks. Hepatic non-heme iron concentrations were measured by colorimetric assays. Protein levels of hepatic ferritin and transferrin receptor were determined by Western blotting. Methylcytosine, hydroxymethylcytosine and unmodified cytosine in DNA were simultaneously measured by liquid chromatography/mass spectrometry method.

Results: Iron supplementation significantly increased hepatic non-heme iron contents ($P < 0.05$) but alcohol alone did not. However, both alcohol and iron significantly increased hepatic ferritin levels and decreased hepatic transferrin receptor levels ($P < 0.05$). Alcohol reduced hepatic DNA hydroxymethylation (0.21% ± 0.04% vs. 0.33% ± 0.04%, $P = 0.01$) compared to control, while iron supplementation to alcohol diet did not change DNA hydroxymethylation. There was no significant difference in methylcytosine levels, while unmodified cytosine levels were significantly increased in alcohol-fed groups compared to control (95.61% ± 0.08% vs. 95.26% ± 0.12%, $P = 0.03$), suggesting that alcohol further increases the conversion from hydroxymethylcytosine to unmodified cytosine.

Conclusions: Chronic alcohol consumption alters global DNA hydroxymethylation in the liver but iron supplementation reverses the epigenetic effect of alcohol.

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Key Words: Alcohols, Iron, DNA hydroxymethylation, DNA methylation, Epigenetics

INTRODUCTION

DNA methylation, methylated cytosine in CpG dinucleotides, has been most extensively studied due to its influence on gene transcription. This characteristic DNA methylation is involved in many physiologic and pathologic processes and is an important etiologic mechanism for the development and progression of cancer. DNA hydroxymethylation is another epigenetic modification of cytosines within DNA, and has recently been the topic of epigenetic studies. Hydroxymethylated cytosines in CpG residues function as an active DNA demethylation step, but also have unique gene regulatory functions that are somewhat

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different from DNA methylation. Furthermore the distribution of DNA hydroxymethylation is more variable in each tissue than that of DNA methylation.

Our previous animal study demonstrated that chronic alcohol consumption at 18% of energy significantly reduced global levels of hepatic DNA hydroxymethylation in young mice but not old ones. This pattern of reduced global hydroxymethylcytosine is similarly seen in cancerous tissue and, therefore, it is plausible that this alcohol consumption may create a tumorigenic environment in the liver, thereby predisposing the tissue to cancer. By better understanding how chronic alcohol consumption may affect global DNA hydroxymethylation we may gain insight into the early stages of this tumorigenic environment.

The process of hydroxylation of methylcytosine is catalyzed by ten-eleven translocation (TET) enzymes, which has been regarded as the first step of active demethylation. In many cancers TET gene mutations and aberrant DNA hydroxymethylation have been found, suggesting that the alteration of DNA hydroxymethylation is critical to carcinogenesis. Interestingly, the conversion of methylcytosine to hydroxymethylcytosine is dependent on iron, a cofactor of TET enzymes, which is an essential nutrient for an array of key biological processes including oxygen transport, cellular respiration through electron transport, DNA replication, DNA repair, and free radical production.

Because alcohol often interferes with iron metabolism, we wanted to investigate whether the influence of alcohol consumption on the hydroxylation could be modulated by iron supplementation. In the present study we attempted to validate the epigenetic effect of alcohol on DNA hydroxymethylation in a different animal model at the different dietary alcohol level and further to demonstrate the effect of iron on alcohol associated epigenetic change.

**MATERIALS AND METHODS**

1. Animal study and diets

Twenty four 8 week old male Sprague-Dawley rats (SLC Inc., Hamamatsu, Japan) were fed one of four different diets: 1) control group, Lieber-DeCarli control diet (0% calorie from ethanol); 2) alcohol group, Lieber-DeCarli alcohol diet (36% calories from ethanol); 3) iron group, Lieber-DeCarli control diet (0% calorie from ethanol) with iron supplementation (0.6% iron); and 4) iron + alcohol group, Lieber-DeCarli alcohol diet (36% calories from ethanol) with iron supplementation (0.6% iron) ($n = 6$ per each group). We chose the dose, 0.6% iron, based on the results from a previous study, which showed a significant interaction between iron and alcohol on liver damage. The alcohol feeding protocol with Lieber-DeCarli alcohol diet is a standard method that has been extensively used as an animal model of alcohol consumption. In particular, the Lieber-DeCarli alcohol diet provides sufficient amount of all essential nutrients in a liquid formula, and successfully induces alcoholic liver disease within 4 to 8 weeks of feeding.

After one-week acclimation on a chow diet, all animals were fed a Lieber-DeCarli liquid diet (Dyets, Inc., Bethlehem, PA, USA) without ethanol for five days. Ethanol was gradually introduced over a 10-day period before providing animals with the final concentration of 6.2% (vol/vol) (36% of total calories as ethanol).

In the control diet, ethanol was replaced by an isocaloric amount of maltodextrin. Rats were killed after 8 weeks of pair feeding and harvested liver tissues were stored at −80°C. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(SE)-09-002).

2. Measurement of hepatic non-heme iron contents and protein levels of ferritin and transferrin receptor

Liver non-heme iron content was measured by colorimetric assay. Briefly, 0.1 g liver tissues were digested in 2 mL acidic solution (3 mole/L HCl and 10% trichloroacetic acid) for 20 hours at 65°C. Digested samples were incubated with chromogen reagent containing 0.1% bathophenathrolinesulfonate and 1% thioglycolic acid for 10 minutes at room temperature, and the absorbance at 535 nm was measured by spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). The protein expressions of hepatic ferritin, which stores iron, and transferrin receptor, which imports iron into the cell, were measured using the western blotting according to a standard procedure. Tissue level of ferritin is known to be positively associated with iron, whereas tissue levels of transferrin receptor are negatively associated with that of iron. Liver tissue is dissolved in the lysis buffer containing 1 mmol/L phenylmethanesulfonylfluoride (25 mmol/L Tris-HCl, 1% NP-40, 1% sodium deoxycholate, 150 mmol/L NaCl, 1% SDS) and added protease inhibitors. After centrifuge protein levels of supernatants were determined by Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) and protein extracts were separated on the 10% SDS PAGE gel. Primary antibodies used in this study were ferritin (The Binding Site Group Ltd., Birmingham, UK), transferrin receptor (Thermo Fisher Scientific, Bremen, Germany) and beta-actin (Santa Cruz Biotechnology, Dallas, TX, USA) as a loading control. Band densities were quantified by using a chemi-doc imaging system (Clinx Science Instruments Co., Shanghai, China).
3. Measurement of methylcytosine, hydroxymethylcytosine and unmodified cytosine

Extraction of DNA was conducted using the conventional phenol/chloroform/isooamyl alcohol method. Hydrolysis of DNA was performed as previously described. In brief DNA was hydrolyzed to nucleosides using nuclease P1, phosphodiesterase I and alkaline phosphatase (all from Sigma, St. Louis, MO, USA). Thereafter, internal standards were added to samples. The isotope-labeled internal standard for deoxycytidine was [\(^{15}\)N]2′-deoxycytidine, while that of 5-methyl-deoxycytidine and 5-hydroxymethyl-deoxycytidine were (methyl-d3,ring-6-d1)-5′-methyl-2′-deoxycytidine (both from Cambridge Isotopes Laboratories, Inc., Andover, MA, USA). The DNA hydrolysates were separated by an Agilent 1100 high-performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) and masses were detected through a 3200 Q Trap MS-MS system (Applied Biosystem, Concord, ON, Canada). Using the known masses of isotope-labeled internal standards added to each sample and the area of each peak the absolute amount of unmodified cytosine, 5′-methylcytosine and 5′-hydroxymethylcytosine per 1 μg of DNA.
can be calculated. Thereafter each amount was expressed as a percentage of total cytosine as we previously described.  

4. Statistics

Differences in percent methylation, hydroxymethylation and unmodified cytosine were determined through a two-way ANOVA followed by a Tukey-Kramer adjustment for multiple comparisons. Hepatic non-heme iron contents and protein levels of ferritin and transferrin receptor were compared by one-way ANOVA followed by Duncan’s multiple range tests. Differences were considered statistically significant at a $P < 0.05$. Data were analyzed using SAS ver. 9.3 (SAS Institute. Cary, NC, USA).

RESULTS

1. Hepatic non-heme iron levels and protein expression of hepatic ferritin and transferrin receptor

Hepatic non-heme iron contents, expressed either $\mu$g/mg protein (Fig. 1A) or mg/total liver (Fig. 1B), were significantly increased in the iron supplemented groups regardless of alcohol consumption, while alcohol consumption alone did not increase hepatic non-heme contents. On the other hand, all three diet groups showed significantly increased ferritin levels compared to the control group (Fig. 2A) and decreased transferrin receptor levels (Fig. 2B) ($P < 0.05$), indicating that chronic alcohol consumption and iron supplementation, both individually and combined, modify the proteins of which gene expression is responsive to cellular iron contents.

2. DNA methylation and hydroxymethylation as well as unmodified cytosine

There were no significant changes in DNA methylation among any of the diet groups (Fig. 3); however, significant changes occurred in the percent of cytosines that were hydroxymethylated. When determining the effect of alcohol alone on percent hydroxymethylation regardless of whether the rats were also fed an iron supplemented diet, we found a significant decrease in the livers of rats fed an alcohol-containing diet (Fig. 4) (0.21% ± 0.04% vs. 0.33% ± 0.04%, $P = 0.01$; Tukey-Kramer Adjustment). Furthermore, when the data is broken up into four groups to include the effect of iron and alcohol, a significant decrease in DNA hydroxymethylation was seen in rats fed the alcohol diet relative to the control group (0.30% ± 0.02% vs. 0.37% ± 0.04%, $P = 0.03$) (Fig. 5). Rats that were fed the iron + alcohol diet had a percent hydroxymethylation that was comparable to both the rats in the control or iron diet group.

Alcohol increased the percentage of cytosines that were unmodified, regardless of iron supplementation (95.61% ± 0.08% vs. 95.26% ± 0.12%, $P = 0.03$) (Fig. 6). Alcohol tended to increase the percentage of cytosine that was unmodified in both the control and the iron supplemented groups, though neither of these results reached significance (Fig. 7).
Figure 5. Influence of iron supplementation on DNA hydroxymethylation reduced by chronic alcohol consumption. A significant decrease in DNA hydroxymethylation was seen in rats fed the alcohol diet without iron relative to the control group ($P = 0.03$). When rats were fed a diet containing both alcohol and iron, the percent hydroxymethylation was equivalent to the rats fed a control diet with or without iron. Values are averages and SE (n = 6 per group). C, Lieber-DeCarli control diet (0% calorie from ethanol); A, Lieber-DeCarli alcohol diet (36% calories from ethanol); Fe, Lieber-DeCarli control diet (0% calorie from ethanol) with iron supplementation (0.6% carbonyl iron); Fe + A, Lieber-DeCarli alcohol diet (36% calories from ethanol) with iron supplementation (0.6% carbonyl iron). *Statistically significant at a $P < 0.05$.

Figure 6. Influence of chronic alcohol consumption on unmodified cytosine levels. The level of unmodified cytosine level was significantly increased in the livers of rats fed an alcohol-containing diet compared to control regardless of iron supplementation ($P = 0.03$). Values are averages and SE (n = 12 per group). C, Lieber-DeCarli control diet (0% calorie from ethanol); A, Lieber-DeCarli alcohol diet (36% calories from ethanol). *Statistically significant at a $P < 0.05$.

Figure 7. Influence of chronic alcohol consumption and iron on unmodified cytosine levels. There was no significant difference in unmodified cytosine among 4 groups ($P = NS$). Values are averages and SE (n = 6 per group). C, Lieber-DeCarli control diet (0% calorie from ethanol); A, Lieber-DeCarli alcohol diet (36% calories from ethanol); Fe, Lieber-DeCarli control diet (0% calorie from ethanol) with iron supplementation (0.6% carbonyl iron); Fe + A, Lieber-DeCarli alcohol diet (36% calories from ethanol) with iron supplementation (0.6% carbonyl iron).

**DISCUSSION**

The chronic consumption of alcohol significantly decreased hepatic DNA hydroxymethylcytosine. An interesting finding here is that the supplementation of iron into a diet containing alcohol seems to reverse the reduction of hydroxymethylcytosine. Because iron is a cofactor for the TET enzyme catalysis of methylcytosine to hydroxymethylcytosine, this increase in iron loading in the liver would seemingly increase the enzymatic reaction.\textsuperscript{21,22} There is, however, the possibility that the binding constant of iron to the TET enzymes is low enough that the iron-TET binding was saturated in the absence of alcohol, because the level of DNA hydroxymethylation in the liver of rats fed an iron containing diet without alcohol is not significantly different from that of control diet group.

We also show here that rats fed a diet containing alcohol had a significant increase in the portion of cytosines that were unmodified. Previous studies have reported a significant decrease in DNA methylation in alcohol models, and this increase in unmodified cytosine may coincide with those reports, even though the decrease in methylcytosine here does not reach significance.\textsuperscript{23-25} It appears that alcohol reduces DNA hydroxymethylation by increasing the degradation process of hydroxymethylcytosine to cytosine. In fact TET enzymes not only catalyze the reaction from methylcytosine to hydroxymethylcytosine but also catalyze the reactions from hydroxymethylcytosine to formylcytosine as well as from formylcytosine to carboxycytosine, which is finally converted to unmodified cytosine.\textsuperscript{26}

Ferritin, the major iron storage protein composed of multimeric H and L subunits, is transcriptionally and post-transcriptionally up-regulated under iron loading.\textsuperscript{27} In this study, alcohol consumption was associated with a decrease in DNA hydroxyme-
thylation, and the supplementation of iron in the diet blocked this alcohol-associated decrease in DNA hydroxymethylation. Conversely, both the alcohol group and the iron + alcohol group had increased ferritin protein levels. Thus our data suggest that iron itself, rather than iron-bound ferritin, modulates the degree of DNA hydroxymethylation. Future study is needed to confirm this hypothesis.

Taken together, the results presented here validate our previous finding that chronic alcohol consumption decreases the percentage of total cytosine that is hydroxymethylated in hepatic DNA. Even though our previous study took place in mice, the amount of alcohol fed to the mice was lower (18% of total calories derived from ethanol, compared to the 36% of total calories here), and the duration of the study was shorter (5 weeks vs. 8 weeks), the end results were similar. Interestingly, we only saw a decrease in hydroxymethylated DNA in the young mice fed alcohol in our previous study. The mice were 5.5 months old at the end of the study, which is a similar age as the rats here (4 months old), which aided in the validation of our previous findings.

While this data is intriguing, without gene specific techniques, such as a microarray, it is unknown whether this decrease in hydroxymethylcytosine is occurring at genes that may promote tumorigenesis. It is interesting that a similar decrease in hydroxymethylation is shown in cancer, particularly in alcohol associated hepatocellular carcinoma. Through microarray and sequencing technologies, the role of hydroxymethylcytosine in the normal and diseased cell states can be further delineated. Other limitations of the study include the lack of TET enzyme activities, which might further explain the molecular mechanisms by which iron reverses the epigenetic effect of alcohol.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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