

# The Interaction of Alcohol and Iron-Overload in the in-vivo Regulation of Iron Responsive Genes

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## ABSTRACT

Patients with alcoholic liver disease (ALD) frequently exhibit iron overload but the molecular mechanisms of alcohol and iron interaction are still unclear. This study investigated the role of alcohol in the regulation of iron-responsive genes, hepcidin, hemojuvelin and leap-2 in both dietary and genetic mouse models. Ethanol significantly down-regulated liver hepcidin and hemojuvelin gene expression. However, leap-2 gene expression was increased in mice treated with 10 % and 20 % ethanol. We further studied the combined effect of alcohol and iron on the regulation of iron metabolism. Iron overload up-regulated the expression of hepcidin, hemojuvelin and leap-2 genes. Interestingly, ethanol reversed the effect of iron on hepcidin and leap-2 gene expression. This effect of ethanol was evident in both dietary and genetic iron overload models. However, further treatment with ethanol did not alter the effect of iron on leap-2 gene expression. Moreover, ethanol and iron together resulted in fatty liver, which was exacerbated with an increasing ethanol concentration. However, mice treated with alcohol or iron alone did not exhibit lipid vesicles in the liver. Collectively, these results suggest that alcohol alters the regulation of iron-responsive genes leading to abnormal iron homeostasis, which may play a role in the progression of alcoholic liver disease.

Keywords: *alcohol liver disease, hepcidin, hemojuvelin, leap-2, alcohol and iron-overload*

## INTRODUCTION

Alcoholic Liver disease (ALD) is a major cause of death in the United States of America and worldwide. ALD is defined by fat accumulation in the liver, hepatitis, and fibrosis, which eventually leads to cirrhosis and hepatocellular carcinoma.<sup>1</sup> Oxidative stress plays an important role in the pathogenesis of ALD and is mainly caused by the generation of free radicals (superoxide hydrogen peroxide, and hydroxyl radical).<sup>2</sup> Patients with ALD frequently exhibit iron overload but the molecular mechanisms of alcohol and iron interaction are still unclear.<sup>3,4</sup>

Iron plays an important role in various essential cell functions. However, excess iron is toxic and causes lipid peroxidation and tissue damage. Its absorption and transport therefore needs to be tightly regulated. Approximately one third of total body iron is bound to storage proteins (ferritin or hemosiderin) and the other two thirds of total body iron is involved in metabolic or enzymatic functions.<sup>5</sup> Iron is needed for essential cell functions such as DNA synthesis, transport of oxygen and electrons, and cell respiration.<sup>6</sup> Dietary iron is absorbed through the duodenum and excess iron is stored in macrophages and the liver.

Recently various iron-regulatory genes have been identified. Hepcidin, synthesized by the liver, is a circulatory peptide, which plays an essential role in the regulation of iron homeostasis.<sup>7,8</sup> Leap-2 peptide, which has similar structure to hepcidin is also synthesized in the liver as an antimicrobial peptide. However, its role in iron homeostasis is unknown. Hemojuvelin is a glycosylphosphatidylinositol-

anchored membrane protein which plays a role in the regulation of hepcidin gene expression in the liver.

Iron is considered to be a secondary risk factor in the progression of ALD. Even moderate alcohol consumption leads to increased serum iron indices.<sup>4</sup> Recently, studies from this laboratory demonstrated a role for alcohol metabolism-mediated oxidative stress in the regulation of hepcidin gene expression.<sup>9</sup> However, the molecular mechanisms of how alcohol and iron interact require further investigation.

## MATERIALS AND METHODS

### Experimental Animal Models

*Dietary iron overload model:* 129/Sv mice (30g initial weight) were fed with custom-prepared (Bio-Serv Labs., USA) egg-white-based iron deficient (0.02% carbonyl iron) diet for 1 week, followed by an iron-overload (2% carbonyl iron) diet for 3 weeks. Subsequently, these mice were treated with either ethanol (10 or 20 %) in the drinking water or plain water (control) for another week. The mice were then sacrificed and organs harvested for further analysis.

*Genetic iron overload model:* Genetic hemochromatosis gene, Hfe knockout mice supplied by Dr. N. Andrews (Harvard Univ., Boston) were fed either with 10 % ethanol in drinking water or plain water for 1 week. Mice were subsequently sacrificed to harvest organs.

### RNA Isolation

The liver specimens were washed with PBS lysed

in Trizol (Invitrogen Corporation) to isolate RNA according to the manufacturer's specifications.<sup>9</sup>

### cDNA Synthesis

cDNA is a DNA copy synthesized from mRNA. The procedure uses 1- 5 µg RNA, 2.5 µM random hexamers (Applied Biosystems), and 200 units of Superscript II reverse transcriptase (Invitrogen Corporation) were employed to synthesize cDNA.<sup>9</sup>

### Real-time Quantitative PCR

cDNA was employed as a template to detect gene expression by using Taqman florescent probes (5'FAM; 3' Blackhole) and an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Control samples were used to eliminate possible genomic DNA contamination by omitting the reverse transcriptase enzyme when making the cDNA. The sequences of probes and sense and antisense primers are shown in Table 1.<sup>9</sup> Data analysis of the endogenous control (gapdh) gene and the target gene cycle numbers was analyzed using Sequence Detection Systems software (Applied Biosystems). Quantification of the amplified control and target genes can be calculated using the kinetic curves.<sup>10</sup>

### Liver Histology and Iron Concentration

Liver tissue samples were taken from each experimental group of mice and the samples were fixed in neutral-buffered 10% formalin. Routine histology processing of the liver tissue was performed by Dr. Dee Harrison Findik.<sup>9</sup> Liver specimen histology was determined by staining the samples with hematoxylin and eosin (H & E). Liver specimens were also stained with Perl's Prussian blue stain to semi-quantitatively determine liver iron content using light microscopy.<sup>9</sup>

### Statistical Analysis

Data points of all gene concentrations were compiled and organized using Excel. Graphs were made using Excel to analyze the data and the Spearman correlation coefficient (referred to as "r" and ranging from -1 to 1) was determined for correlation analysis. To determine the differences in the different treatment groups the analysis of variance (ANOVA) was performed.

## RESULTS

Analysis of the experiment which employed 129/Sv mice fed with iron deficient (control) or iron deficient and ethanol (10% or 20%) diets showed there was a down-regulation in hepcidin (Hepc) by 2.2-fold ( $0.449 \pm 0.05$ ) with the 10% and 3.4-fold ( $0.29 \pm 0.09$ ) with the 20% ethanol treatment (Fig. 1). These results confirmed the previous work done in the lab. The Gene Hemojuvelin, also analyzed in the experiment, contained similar results with the 20% ethanol treatment because it was also downregulated by 2.5-fold ( $0.4 \pm 0.17$ ) however the 10% ethanol treatment is questionable and could be independent. Hemojuvelin is not a well known and analyzed gene in the liver, but it is thought to be upstream of hepcidin and regulate hepcidin by certain stimuli. In contrast, in this experiment, Leap-2 mRNA expression was up-regulated by 3.5-fold ( $3.57 \pm 0.59$ ) in the 10% ethanol treatment and by 2.7-fold ( $2.66 \pm 0.3$ ) in the 20% ethanol treatment. Leap-2 is also not a very well known gene however it is very similar in composition to hepcidin.

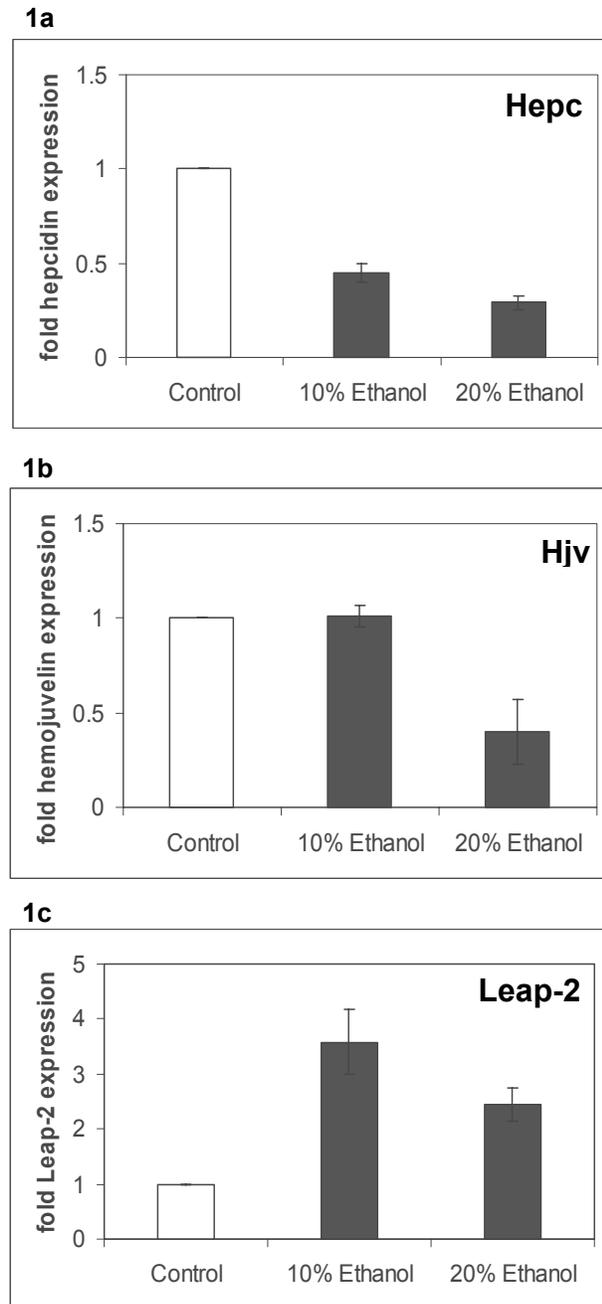
### Dietary Iron Overload

Data collected from the second experiment employing the combined effect of iron and ethanol on mRNA expression of hepcidin, hemojuvelin, and leap-2 genes in 129/Sv mice fed with iron deficient (control), iron overload (iron) or iron overload and ethanol (iron + 10%, and iron + 20%) was detected using the same method (Fig. 2). In the dietary iron overload control mice hepcidin ( $2.8 \pm 0.6$ ), hemojuvelin ( $4.22 \pm 1.39$ ) and leap-2 ( $3.102 \pm 1.9$ ) mRNA expression was up-regulated. However, with the iron and ethanol fed mice they together resulted in a significant (Students paired T-test of  $p < 0.01$ ) down-regulation of hepcidin (iron + 10% =  $1.58 \pm 0.9$  and iron + 20% =  $2.28 \pm 0.39$ ) and hemojuvelin (iron + 20% =  $1.96 \pm 0.029$ ) gene expression compared to the iron overload alone. Interestingly, iron and 10% ethanol treatment resulted in mild steatosis (fatty liver), which was more prominent with the 20% ethanol exposure (Fig. 3). This could explain why the levels of hepcidin in the iron and 20% mice were higher due to liver damage which can lead to altered

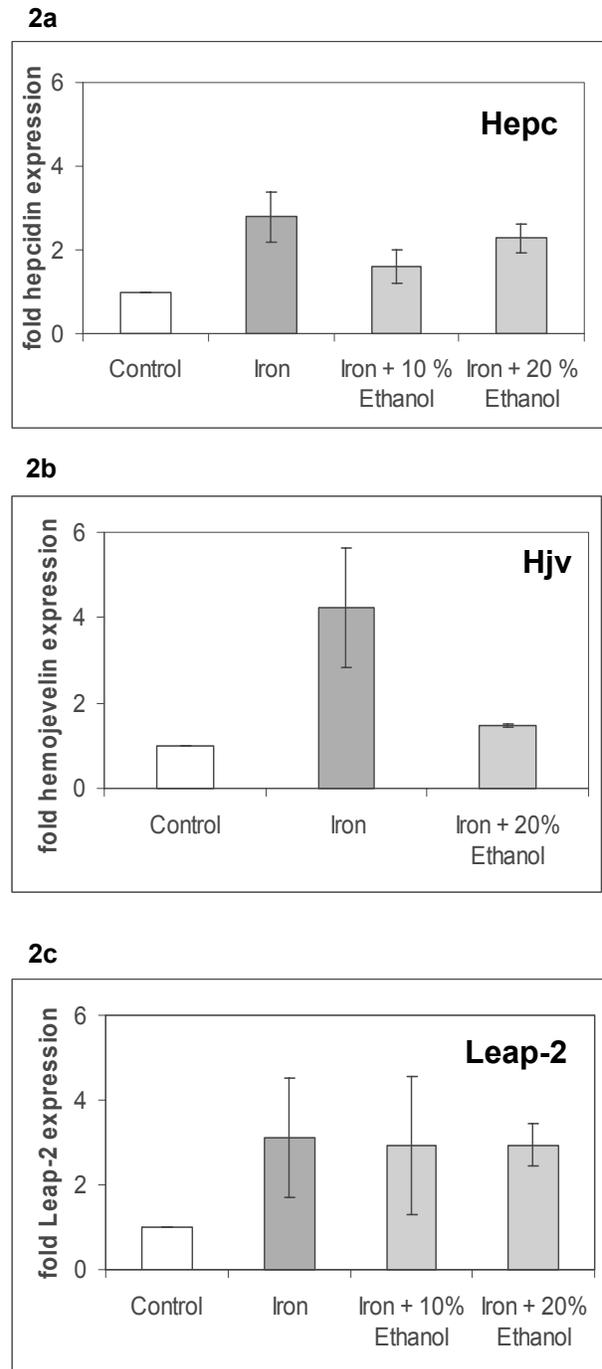
| Mouse Gene         | Forward Primer 5'-3'         | Reverse Primer 5'-3'           | Taqman Probe 5'-3'               |
|--------------------|------------------------------|--------------------------------|----------------------------------|
| <b>Hepcidin1</b>   | TGCAGAAGAGAAGGAAG<br>AGAGACA | CACACTGGGAATTGTTACAGCAT<br>T   | CAACTTCCCCATCTGCATCTTCTGC<br>TGT |
| <b>LEAP-2</b>      | GGGTTTCCCTCAGGCC             | TGCATAGTCTTGTGATACTCA<br>GAGTC | TTGGTGCCTCGTGCCGGGA              |
| <b>Hemojuvelin</b> | TCTCCTTCTCCATCAGGGT<br>AGC   | TGTAGGTCCTGCTCTGCGG            | AGGATGTGGCGGGGCTTC               |
| <b>GAPDH</b>       | TCACTGGCATGGCCTTCC           | GGCGGCACGTCAGATCC              | TTCCTACCCCCAATGTGTCCGTCG         |

**Table 1:** Sequence of probe and sense and antisense primers used during Real-time quantitative PCR

regulation of hepcidin. This was not observed in mice treated with either ethanol or iron alone. Iron overload and ethanol, together, up-regulated leap-2 (iron + 10% =  $2.92 \pm 1.69$  and iron + 20% =  $2.93 \pm 0.50$ ) gene expression compared to the iron overload alone.

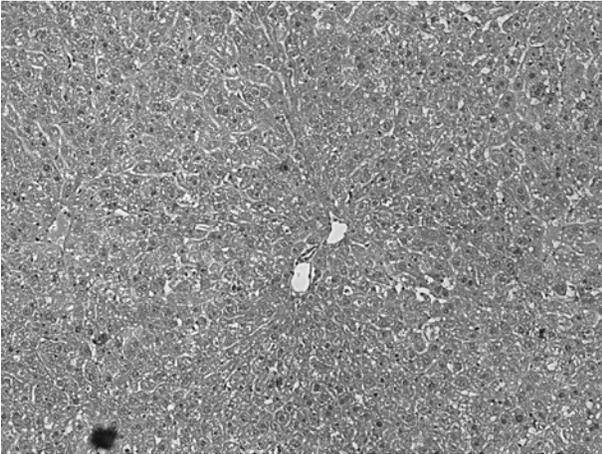


**Figure 1(a,b,c):** The effect of ethanol on mRNA expression of hepcidin (Hepc), hemojuvelin (Hjv), and leap-2 genes in 129/Sv mice fed with iron deficient (control) or iron deficient and ethanol (10% or 20%) diets has been detected with real-time PCR, as described in Methods.

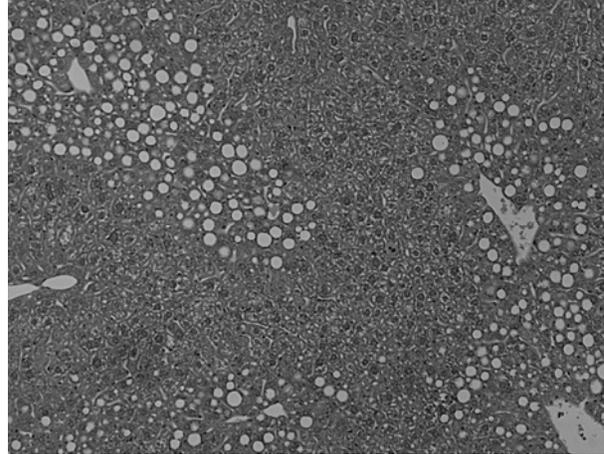


**Figure 2 (a,b,c):** The combined effect of ethanol on mRNA expression of hepcidin (Hepc), hemojuvelin (Hjv), and leap-2 genes in 129/Sv mice fed with iron deficient (control), iron overload (iron) or iron overload and ethanol (iron + 10%, iron + 20%) diets has been detected with real-time PCR.

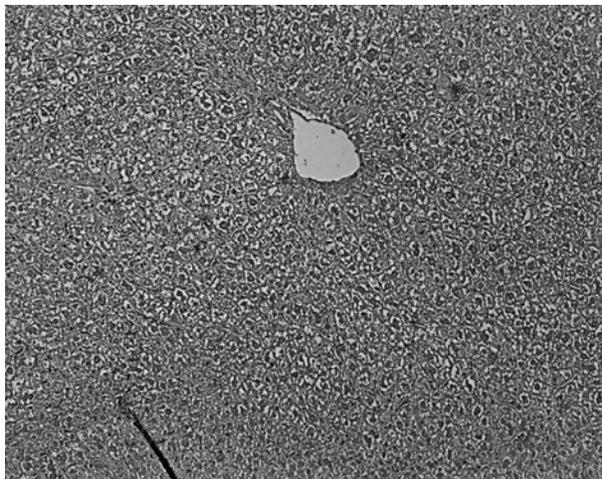
3a



3d



3b

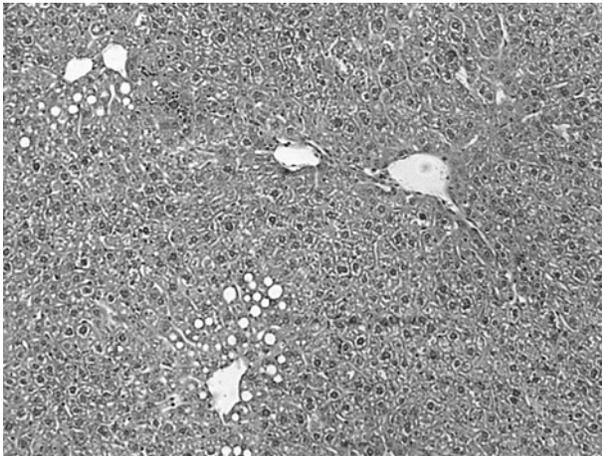


**Figure 3(a,b,c,d):** Hematoxylin and Eosin staining of liver tissue from mice fed with (a) iron deficient, (b) iron overload, (c) 10% ethanol and iron and (d) 20% ethanol and iron diet, as described in Methods (10 x magnification).

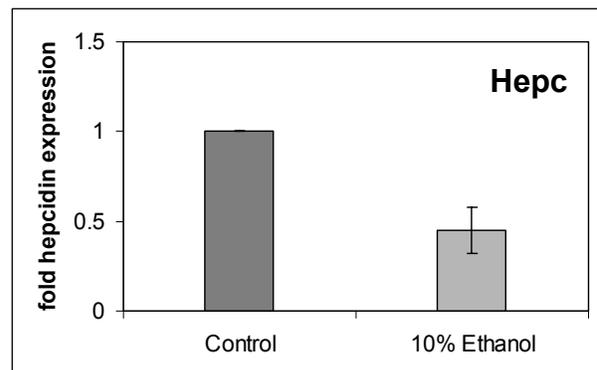
#### Genetic Iron Overload

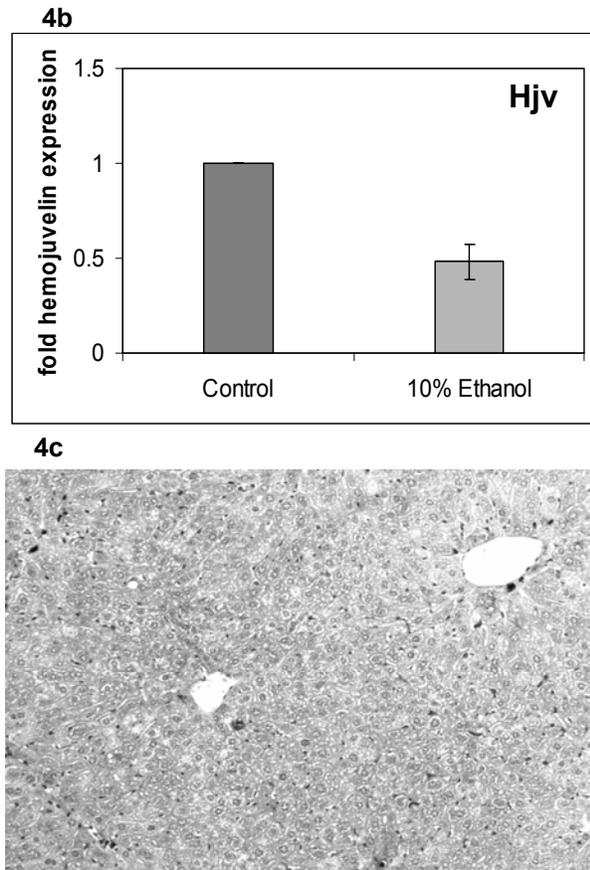
Human iron overload disorder, genetic hemochromatosis (GH) is prominent in the Caucasian population. It is caused by mutations in the Hfe gene. However, the function of this gene is unknown. The Hfe knockout mice are an animal model for this disease. Both in GH patients and in the Hfe knockout mice hepcidin is downregulated despite the iron overload. Normally, in iron overload hepcidin in the liver is increased. In this experiment the Hfe knockout mice were exposed to 10% ethanol and the results displayed a 2-fold down-regulation of both liver hepcidin and hemojuvelin genes compared to the untreated (control) mice (Fig. 4).

3c



4a





**Figure 4 (a,b,c):** The effect of ethanol on (a) hepcidin (Hepc) and (b) hemojuvelin (Hjv) mRNA expression in the liver of HFε Knockout mouse. (c) Iron Staining (Prussian Blue).

## DISCUSSION

In Conclusion and iron overloaded diet up-regulates the expression of the iron-responsive genes, hepcidin, hemojuvelin, and leap-2. Similarly to the iron, alcohol up-regulates leap-2 gene expression. However, in contrast to the iron, alcohol down-regulates both liver hepcidin and hemojuvelin gene expression. Moreover, ethanol nullifies the effect of both dietary and genetic iron overload on hepcidin and hemojuvelin gene expression. Interestingly, further treatment with ethanol does not alter the effect of iron on leap-2 gene expression. Treatment with iron and ethanol results in fatty liver. Collectively alcohol regulates iron metabolism, which may play a role in the progression of alcoholic liver disease.

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