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Ethanol affects hepatitis C pathogenesis: Humanized SCID Alb-uPA mouse model

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ABSTRACT

Alcohol consumption exacerbates the course of hepatitis C viral (HCV) infection, worsens outcomes and contributes to the development of chronic infection that exhibits low anti-viral treatment efficiency. The lack of suitable *in vivo* models makes HCV–ethanol studies very difficult. Here, we examine whether chimeric SCID Alb-uPA mice transplanted with human hepatocytes and infected with HCV develop worsening pathology when fed ethanol. After 5 weeks of feeding, such mice fed chow + water (control) or chow + 20% ethanol in water (EtOH) diets mice developed oxidative stress, decreased proteasome activity and increased steatosis. Importantly, HCV^+ mice in the control group cleared HCV RNA after 5 weeks, while the infection persisted in EtOH-fed mice at the same or even higher levels compared with pre-feeding HCV RNA. We conclude that in chimeric SCID Alb-uPA mice, EtOH exposure causes the complex biochemical and histological changes typical for alcoholic liver injury. In addition, ethanol feeding delays the clearance of HCV RNA thereby generating persistent infection and promoting liver injury. Overall, this model is appropriate for conducting HCV–ethanol studies.

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1. Introduction

Ethanol consumption exacerbates hepatitis C virus (HCV)-infection pathogenesis and decreases the sensitivity of HCV patients to anti-viral treatment. However, the mechanisms of ethanol–HCV interactions are not clear yet. Moreover, the lack of the adequate animal models makes conducting such studies extremely difficult since HCV is a human virus, which does not infect the rodents. *In vitro* ethanol studies using hepatoma cells of Huh 7.5 origin are not always successful because these cells while allowing viral replication do not express ethanol-metabolizing enzymes, alcohol dehydrogenase and cytochrome P450 2E1, and do not generate the most toxic ethanol metabolites. Thus, animal studies are critical for examining the true effects of ethanol on HCV pathogenesis. The most attractive way to solve this problem is using chimeric mice with transplanted human hepatocytes, which can be naturally infected with HCV.

Currently, several available mouse strains (SCID Alb-uPA mice, *Fah*-/-Rag2-/-cg-/-mice, uPA-NOG and TK-NOG) allow up to 70–90% reconstitution of mouse hepatocytes by human ones.

Various strategies were used for the robust depletion of mouse liver cells, including transgenic expression of liver damaging enzyme, murine plasminogen urokinase genes, under the control of the albumin promoter (Alb-uPA) on CB-17-*scid*-bg or NOD-*scid*-γ-/- (NOG) background [1,2], enzymatic deficiency of tyrosine catabolic enzyme fumarylacetoacetate hydrolase (*Fah*) mutants [3], conditional depletion of mouse hepatocytes expressing FK506 binding protein (FKBP)-caspase 8 fusion gene driven by the albumin enhancer/promoter (AFC8) during first week after birth [4] and depletion of hepatocytes in adult mice carrying herpes simplex virus thymidine kinase by gancyclovir [5]. Some of these mice were reported to be infected with HCV [1,6,7].

Abbreviations: HCV, hepatitis C virus; EtOH, ethanol; ALT, alanine aminotransferase; TBARS, thiobarbituric acid-reactive substances; proteasome ChT-like activity, chymotrypsin-like activity; PCR, polymerase chain reaction.

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Here, we used HCV+ and HCV− SCID Alb-uPA mice that carry a tandem array of murine urokinase genes under the control of an albumin promoter. Transgene expression accelerates mouse hepatocyte death, thereby leading to re-population (up to 70–80%) of the mouse liver with transplanted human hepatocytes that can be further infected with HCV. The goal of these experiments was to examine whether chimeric HCV-infected and non-infected SCID Alb-uPA mice tolerate chronic ethanol feeding and whether this feeding paradigm induces the typical biochemical and morphological features of alcoholic liver damage.

2. Materials and methods

2.1. Mice

The SCID AIb-uPA mice and transplantation of human hepatocytes were performed as described previously [1]. All mice were transplanted with hepatocytes from the same donor. SCID AIb-uPA mice with significant human repopulation of the liver (defined as serum human albumin level >1 mg/mL at 8–10 weeks after transplantation) were used for the current study. **Human albumin** levels in mouse serum were measured with a human albumin enzyme-linked immunosorbent assay (ELISA) Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX). For quantitative analysis of **HCV RNA**, 100 µl mouse serum samples were used. Total RNA were isolated by MagMAX Viral RNA Isolation Kit (Ambion, AM1939), according to the instructions provided by the manufacturer. Ten µl total RNA were reverse transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). HCV RNA was quantified by real time PCR using Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems. We used standard curve method by OptiQual HCV RNA Control. The lower detection limit of this assay is 4 copies per well.

Mice were either fed chow-pelleted diet and water (control, or H_2O group) or chow-pelleted diet and 20% ethanol in water (EtOH group) for 5 weeks as previously described [8,9]. Each group (control and EtOH in HCV^+ and HCV^- mice) contained three mice. The care, use and procedures performed on these mice were approved by the Institutional Animal Care and Use Committee at the Omaha Veterans Affairs Medical Center and University of Nebraska Medical Center and complied with NIH guidelines and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Oxidative stress-related parameters were examined in total liver homogenates. Oxidant formation was determined by measuring malondialdehyde (MDA) by thiobarbituric acid-reactive substances (TBARS) using a kit (Cayman Chemical Company, Ann Arbor, MI). Antioxidant defense was quantified by measuring glutathione levels using the enzymatic recycling method [10].

Proteasome activity was measured in homogenates prepared from mouse livers. The chymotrypsin-like (ChT-like) peptidase activity of proteasome was detected by *in vitro* Suc-LLVY-AMC fluorometric assay as described [11,12].

Lipid accumulation in the liver was quantified by determining triglyceride levels [13].

Liver histology was estimated using paraffin-embedded tissue, H&E staining.

2.2. Statistical analyses

Data are expressed as mean values ± standard deviation. Comparisons among multiple groups were determined by

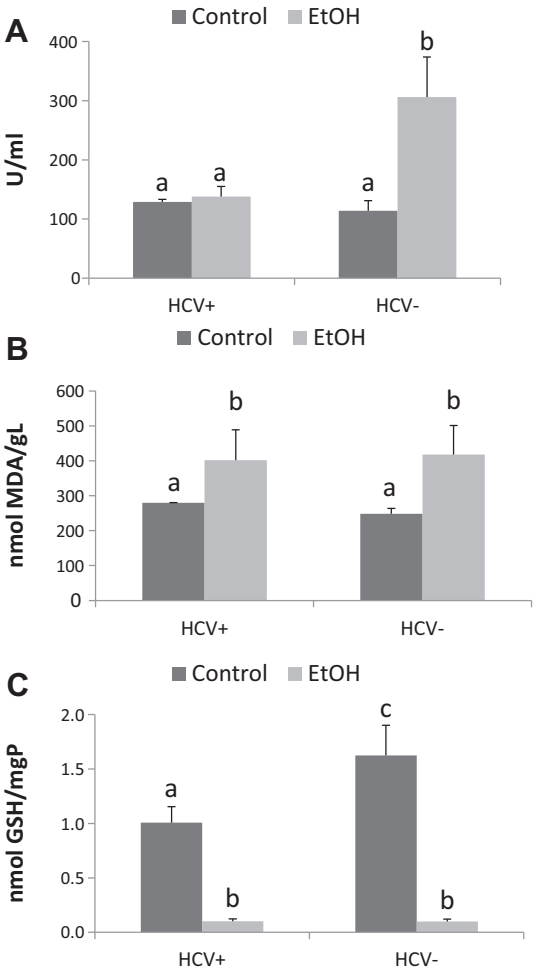

Fig. 1. Biochemical changes in HCV^+ and HCV^- mice fed control or ethanol diet. (A) Serum ALT; (B) Hepatic TBARS and (C) Hepatic GSH levels. All data are presented as mean ± SEM. Values not sharing a common subscript letter are statistically different, $p < 0.05$.

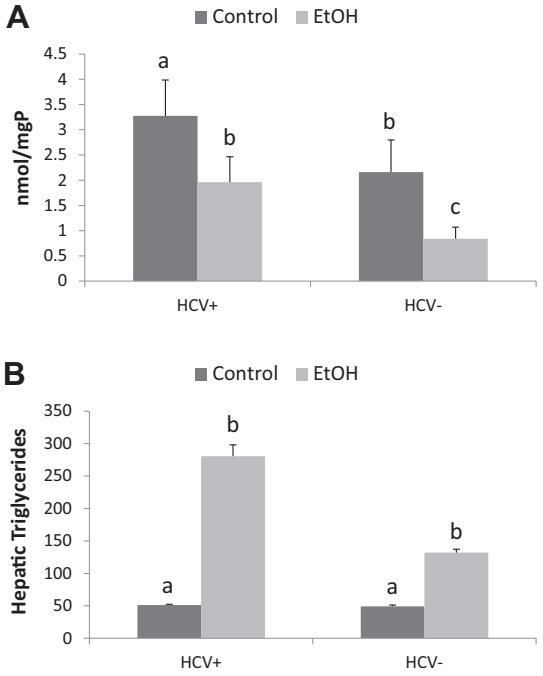

Fig. 2. Hepatic proteasome activity (A) and triglyceride levels (B) in HCV^+ and HCV^- mice fed control or ethanol diet. All data are presented as mean ± SEM. Values not sharing a common subscript letter are statistically different, $p < 0.05$.

Table 1
HCV RNA (copies/ml) before and after ethanol feeding.

Dietary regimen	Pre-feeding	Post-feeding
Ethanol	9.23×10^4	4.76×10^5
Ethanol	1.43×10^3	1.62×10^3
Ethanol	2.22×10^3	1.71×10^4
Control	3.83×10^3	Negative
Control	2.25×10^3	Negative
Control	1.18×10^3	Negative

one-way ANOVA, using a Tukey post hoc test. For comparisons between two groups, we used Student's *t*-test. A probability value of 0.05 or less was considered significant.

3. Results

Both HCV-infected and non-infected mice tolerated ethanol feeding regimen. We observed no difference in ALT levels between HCV^+ control and ethanol-fed mice; however, in HCV^- mice, ethanol feeding induced ALT (Fig. 1A).

Oxidative stress was comparable in HCV^+ and HCV^-mice and increased by ethanol feeding based on elevation of TBARS and reduction in glutathione (GSH) levels (Fig. 1B and C). However, **proteasome activity** (a parameter regulated by oxidative stress) was reduced by 40% in HCV^+ mice and 61% in HCV^- mice by ethanol feeding (Fig. 2A). In addition, **accumulation of triglycerides** was enhanced by ethanol feeding up to 5.5- and 2.7-fold in HCV^+ and HCV^- mice, respectively (Fig. 2B).

HCV RNA titers in mice fed control or ethanol diet are presented in Table 1. All mice from control group cleared up HCV RNA after 5 weeks of feeding, while HCV RNA still persisted and in fact the HCV RNA titer increased after 5 weeks of ethanol feeding,

3.1. Liver histology

Fig. 3 represents hematoxylin–eosin stained images from representative livers of mice transplanted with human hepatocytes, infected or not with HCV and fed water or 20% ethanol in water. Liver sections of the representative HCV^- mice fed water show that the cell size of the transplanted human hepatocytes is almost 3 times larger than the mouse hepatocytes. While ethanol feeding to the HCV-mice induced mild macrosteatosis and ballooning of hepatocytes, these features were enhanced in the HCV^+ mice fed ethanol. In addition, the HCV^+ mice fed ethanol also exhibited cell dysplasia. The HCV^+ mice fed water alone displayed mild macrosteatosis and showed big phagocytic cells that possibly are engaged in scavenging HCV-infected hepatocytes.

4. Discussion

This study was initiated to examine whether immunodeficient (SCID Alb-uPA) mice with transplanted human hepatocytes (a) would tolerate ethanol feeding with 20% ethanol in water; (b) whether such exposure to for 5 weeks would cause the development of biochemical and histological features typical for alcohol liver injury and (c) whether alcohol-induced changes are comparable in HCV-infected and non-infected mice. There are several reasons why ethanol studies were performed in HCV^+ chimeric mice. First, this model is an excellent small rodent model that facilitates HCV replication *in vivo* in ethanol-metabolizing human hepatocytes [14]. Second, the effects of ethanol on HCV-mediated liver injury have not been previously examined in such HCV-infected chimeric mice [6,15,16]. It is vital to examine alcohol–HCV interactions using this mouse model since alcohol exposure potentiates HCV pathogenesis, causing disease exacerbation and affecting outcomes in infected patients [17–19].

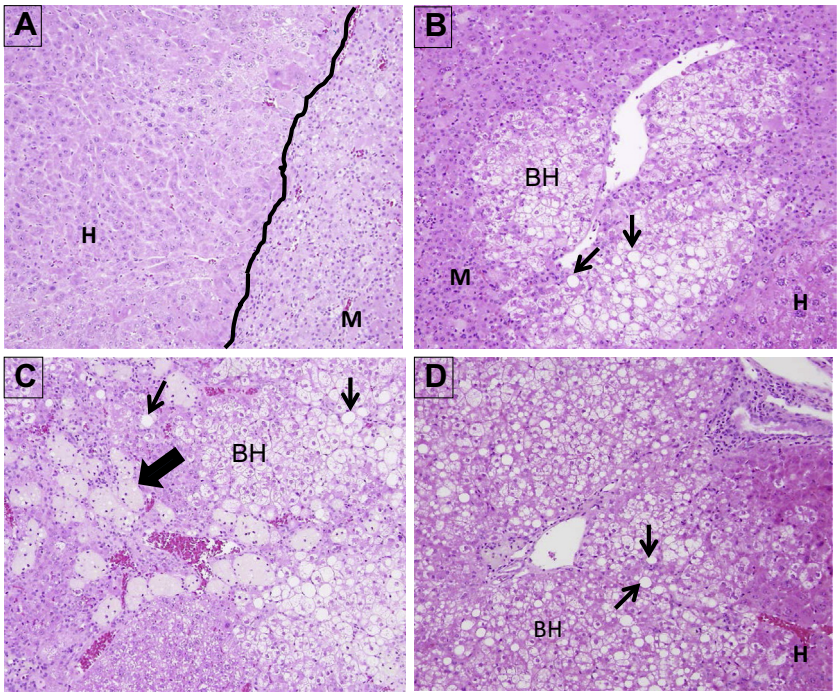

Fig. 3. HCV and ethanol exacerbates liver injury. Hematoxylin–eosin stained images from representative livers of mice transplanted with human hepatocytes and infected or not with HCV and fed water or 20% ethanolic water. (A) HCV^- mice fed water; (B) HCV^- mice fed ethanol water; (C) HCV^+ mice fed water; (D) HCV^+ mice fed ethanol water for 6 weeks. The black line in (A) shows the boundary between the human (H) and the mouse hepatocytes (M). Mild macrosteatosis (arrows); hepatocyte ballooning (BH); clusters of large phagocytic cells (large arrow).

Here, we found ethanol-induced ALT elevation in HCV^- mice, but not in HCV^+ mice, indicating that HCV^+ hepatocytes are more protected from necrotic cell death. Importantly, the lack of correlation between ALT levels and ethanol consumption was also reported in HCV patients [17]. Currently, we can provide no explanation for this phenomenon, since the pattern of changes in glutathione (a major liver antioxidant) levels by ethanol feeding was comparable in HCV^+ and HCV^- animals. In addition, TBARS activity (a pro-oxidative parameter) was equally increased by ethanol in HCV^+ and HCV^- mice, indicating ethanol feeding induces oxidative stress. Thus, the anti-oxidant/oxidant shift induced by feeding ethanol in water is similar in both HCV-infected/non-infected chimeric mice compared with transgenic mice that express either structural or NS5A HCV proteins [9,11]. Nonetheless, the overall oxidative stress seemed to be slightly lower in HCV-infected than in non-infected mice since the proteasome activity that is tightly regulated by oxidative stress levels [18–21] was increased in HCV^+ control mice (usually, it corresponds to low oxidative stress [12]) and then suppressed by ethanol feeding by 40% vs 61% in non-infected mice. However, the changes in proteasome activities by ethanol are not as prominent as observed in HCV transgenic mice [11] that express HCV proteins uniformly as in the SCID Alb-uPA mice, only the transplanted human hepatocytes are infected with HCV.

Another characteristic feature of alcohol-induced liver injury, steatosis, was also induced by ethanol feeding in HCV-infected and non-infected chimeric SCID Alb-uPA mice, based on the elevation of triglyceride levels and liver histology (H&E staining). The magnitude of these changes was more prominent in HCV^+ mice.

Importantly, after 5 weeks of feeding control (chow + water) diet, we detected no HCV RNA in control mice (which were HCV+ at the enrollment). However, ethanol-fed animals expressed measurable HCV RNA after 5 weeks of feeding, indicating that ethanol prevents/slows down HCV RNA clearance.

We conclude that SCID Alb/uPA mice with humanized livers display expected biochemical and histological features as well as persistent HCV RNA following chronic ethanol exposure, and this model is appropriate for conducting HCV–ethanol studies.

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