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Mode of Oral Ethanol Feeding Affects Liver Oxidative Stress Levels and Methylation Status: Study on NS5A-Transgenic Mice

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Authors' contributions

Author Natalia Osna designed the study, performed statistical analysis and wrote the first draft of the manuscript. Author Kusum Kharbanda conduced the HPLC analysis and managed the study analysis. Authors Drs. Poluektova, McVicker and Ganesan provided the data included to the manuscript and managed the literature search. All authors read and approved the final version of the manuscript.

Original Research Article

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ABSTRACT

Background: Alcohol consumption accelerates the progression and worsens the outcomes of hepatitis C viral (HCV) infection in heavy and moderate drinkers. Our aim was to investigate the effects of two modes of oral ethanol feeding on induction of oxidative stress, impaired methylation status and downstream changes in proteasome activity in livers of NS5A-transgenic (Tg) mice.
Methods: Ethanol was administered either in water (chow fed mice given 20% ethanol in water; designated chow-EtOH) or fed in Lieber De Carli liquid diet (LCD-EtOH). Appropriate controls were used. The mechanisms of alcohol and NS5A-induced changes

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in oxidative stress, liver methylation status and 20S proteasome activity were determined after 5 weeks of the feeding regimen.
Results: Ethanol administration using both feeding regimens induced oxidative stress and suppressed cytosolic proteasome activity. However, only LCD-EtOH diet induced fatty changes in the liver which correlated with higher levels of oxidative stress, impaired methylation potential and reduced cytosolic and nuclear proteasome activity. However, LCD diet by itself triggered lipid peroxidation.
Conclusion: We conclude that both modes of oral ethanol feeding (chow and LCD-based) induce oxidative stress in NS5A-Tg mice that suppresses proteasome activity. Nonetheless, impaired methylation potential, higher level of oxidative stress and suppression of nuclear proteasome was observed only in LCD-EtOH mice. However, the effects of LCD-control liquid diet in inducing lipid peroxidation in NS5A-Tg mice, in certain cases, tended to mask the effects of ethanol.

Keywords: NS5A mice; ethanol feeding; oxidative stress; methylation status; proteasome activity.

ABBREVIATIONS

Tg-transgenic; LCD- Lieber De Carli Diet; EtOH-ethanol; ALD- alcoholic liver disease; ALT-alanine aminotransferase; SAM - S-adenosylmethionine; SAH- S-adenosylhomocysteine; HCV-hepatitis C virus; TBARS-thiobarbituric acid-reactive substances; 4HNE-4-hydroxynonenal; ChT-like proteasome activity- chymotrypsin-like proteasome activity; CYP2E1- cytochrome P2E1.

1. INTRODUCTION

Alcohol damages various organs and systems in heavy and moderate drinkers. Since the liver is the major site of ethanol metabolism, alcoholic liver disease (ALD) is a frequently observed in alcoholics. It is accepted that the effects of ethanol on liver pathology is due to its metabolism and dysregulation of multiple signal transduction pathways that subsequently impairs liver cell function [1-6]. *In vivo* consequences of ethanol metabolism are studied on small rodent models. These models usually do not exhibit the whole spectrum of changes typical for alcohol-consuming humans, but there are still certain pathological features that can be induced by ethanol feeding. The expression of these features depends on multiple factors: the strain of mice, their genotypic characteristics (knockouts), nutritional factors, supplements and the mode of alcohol feeding. Here, we compared the effects of two modes of chronic ethanol feeding, ethanol in liquid Lieber DeCarli Diet (LCD) or in drinking water, on features of alcohol-induced liver damage such as necrotic cell death (alanine aminotransferases, ALT), steatosis, oxidative stress and alterations in methylation status .Liver proteasome activity, a parameter that is downstream from oxidative stress and also partially dependent on methylation status (the intracellular ratio of S-adenosylmethionine and S-adenosylhomocysteine, SAM:SAH) was also examined. This study is designed to specifically characterize the advantages and disadvantages of various modes of ethanol feeding that potentially can be used to further investigate proteasome-dependent events, including interferon signaling, antigen presentation and liver fat accumulation in Tg mice expressing hepatitis C viral (HCV) proteins.

As revealed from clinical studies, alcohol tremendously potentiates pathogenesis of HCV-infection [7-9]. Studying interactions between alcohol and viral infection are important. For these studies, we chose NS5A-transgenic mice. NS5A is non-structural HCV protein, which is a part of viral replication complex. Earlier published data on the synergism between HCV and ethanol revealed increased expression of Toll-like receptor 4 in these mice, which makes them highly susceptible to the toxic effects of ethanol [10]. Therefore, NS5A Tg mice is a suitable and a clinically relevant model for conducting such comparative ethanol experiments where differential levels of ethanol metabolites are generated by various modes of oral ethanol feeding. Thus, the objective of this study is to examine the levels of oxidative stress, impaired methylation and downstream changes in proteasome function in NS5A Tg mice fed ethanol either in water or in combination with LCD.

2. MATERIALS AND METHODS

2.1 Mice

We used transgenic FVB mice of mixed genders (about 50% of female and male mice/group), which express NS5A (non-structural HCV) protein (NS5A-tg mice) and were matched in age (3-4 months) and weight (20-25g). Mice were obtained from Dr. Ratna Ray, Saint Louis University. The details about these mice were published elsewhere [11]. Briefly, they were generated by targeting the HCV NS5A genomic region from genotype 1a, cloned under the control of a mouse major urinary promoter in hepatocytes. These mice, expressing HCV NS5A in the liver are phenotypically similar to their normal littermates. The expression of NS5A transgene is comparable to that in HCV-infected liver in humans. NS5A-Tg mice were bred at our VA Animal facility (Omaha, NE) by back-crossing of NS5A+ males and females. 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 complied with NIH guidelines.

2.2 Ethanol Feeding

Ethanol was administered either in water (20% ethanol water, Chow-EtOH) [12] or as part of the Lieber DeCarli liquid diet with 29.2% of calories derived from ethanol (LCD-EtOH) as previously described [13]. The control mice for these two ethanol groups were given either water alone plus LabDiets 5001 Purina chow (Chow-Control) [12,14] or pair-fed the Lieber DeCarli liquid control diet (LCD-Control), respectively. The four experimental groups each consisted of n=7/group. After 5 weeks of feeding, all animals were sacrificed, livers were removed and used for preparing subcellular fractions or immediately clamp frozen in liquid nitrogen and stored at -80ºC till further analysis.

2.3 Subcellular Fractions

(A) Total Liver homogenates: Liver pieces were homogenized in cold Phosphate buffered saline (PBS).
(B) Cytosolic fraction: Total liver lysates were centrifuged at 105,000g for 60 min at 4ºC to yield the cytosol fractions (supernatants).
(C) Nuclear fractions: were obtained from livers according the method of Andrews [15].

2.4 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), 4-hydroxynonenal (4HNE) adducts were measured by Western blot analysis (anti-HNE antibody was from Cell Biolabs, Inc). Antioxidant defense was quantified by measuring glutathione levels using the enzymatic recycling method [16].

2.5 Cellular Methylation Potential

Cellular Methylation Potential Was measured by determining hepatic SAM and SAH levels by HPLC analysis [17].

2.6 Lipid Accumulation

Lipid Accumulation In the liver was measured by determining triglyceride levels [17] and H&E staining. Cytoplasmic lipid droplets (LDs) were visualized by BODIPY staining of fresh frozen (OCT) liver sections.

2.7 Proteasome Activity

Proteasome Activity was measured in cytosolic and nuclear fractions 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 [18].

2.8 Western Blot Analysis

Expression of NS5A protein in liver lysates was confirmed with antibody to NS5A protein (rabbit polyclonal, Abcam) using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

2.9 Statistical Analyses

Data are expressed as mean values±SEM. Comparisons among multiple groups were determined by one-way ANOVA, using a Tukey post-hoc test (Prism Graphpad software 5.01version). For comparisons between two groups, we used Student's t-test. A probability value of .05 or less was considered significant.

3. RESULTS

3.1 ALT, triglyceride Levels, Steatosis in H&E Staining, BODIPY Staining in Ethanol-fed and Control NS5A-Tg Mice

3.1.1 Serum ALT

Serum ALT levels were unchanged in chow–EtOH-fed mice compared with their -chow-fed controls. However, there was a threefold increase in ALT level in LCD-EtOH mice compared with the pair-fed LCD-Control mice (Fig.1A).

3.1.2 Steatosis

There were no differences in triglyceride levels between the chow-control-fed and chow-ETOH-fed groups. However, 2-fold higher level of triglycerides was observed in mice fed LCD-Control diet compared with Chow-Control mice. The mice fed the LCD-EtOHdiet exhibited a further increased triglyceride levels by 1.4-fold over pair-fed controls group (Fig. 1B). The latter triglyceride determinations were consistent with the histological evaluation of steatosis by H & E staining of liver sections (no steatosis in chow-control and EtOH group, low level of steatosis in LCD control group and profound steatosis in LCD-ethanol group,(Figs 2A, B, C, D).

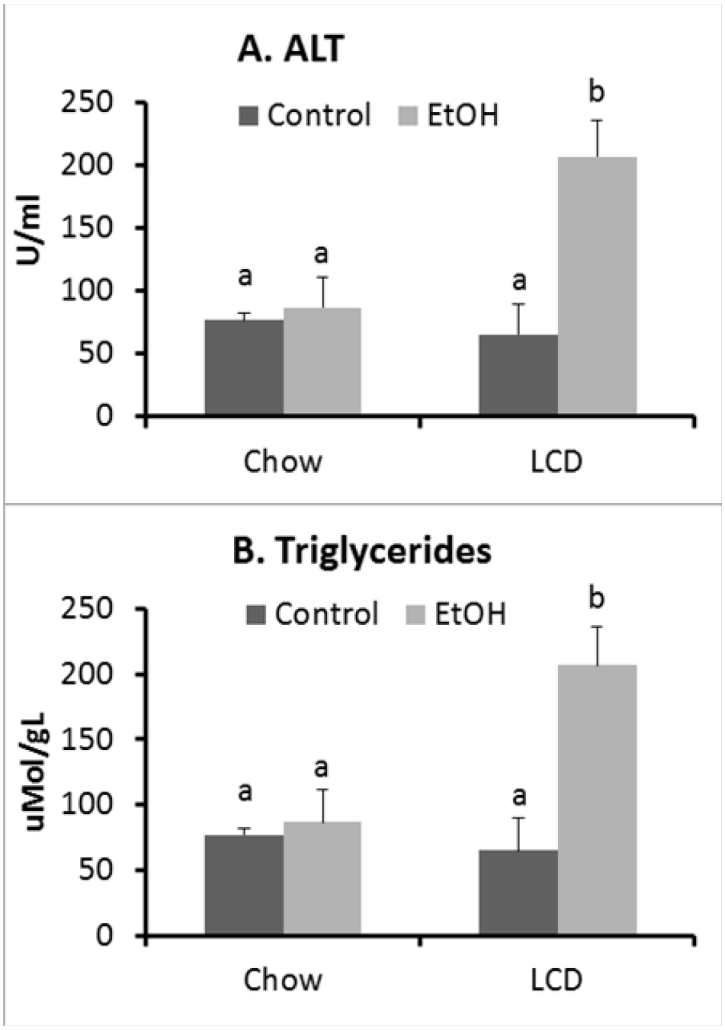

Fig. 1. ALT and triglyceride levels in chow and LCD mice fed control (chow- and LCD-Con) and ethanol (chow-and LCD-EtOH) diets. (A) Serum ALT levels and (B) Liver triglyceride levels were determined as described in Method Section. All data are presented as Mean ±SEM .Values not sharing a common subscript letter are statistically different, (A) a vs b, P=0.019, (B) a vs b. P=0.031

Since ethanol administration in water revealed no changes in the liver steatosis assessed biochemically (triglyceride levels) or by histological evaluation (H&E staining), we performed BODIPY staining only in LCD-fed mice (Figs. 2E and F). LDs were observed in both LCD-Control EtOH-fed NS5A-Tg mice. However, the size of some LDs that was larger in LCD-EtOH-fed mice.

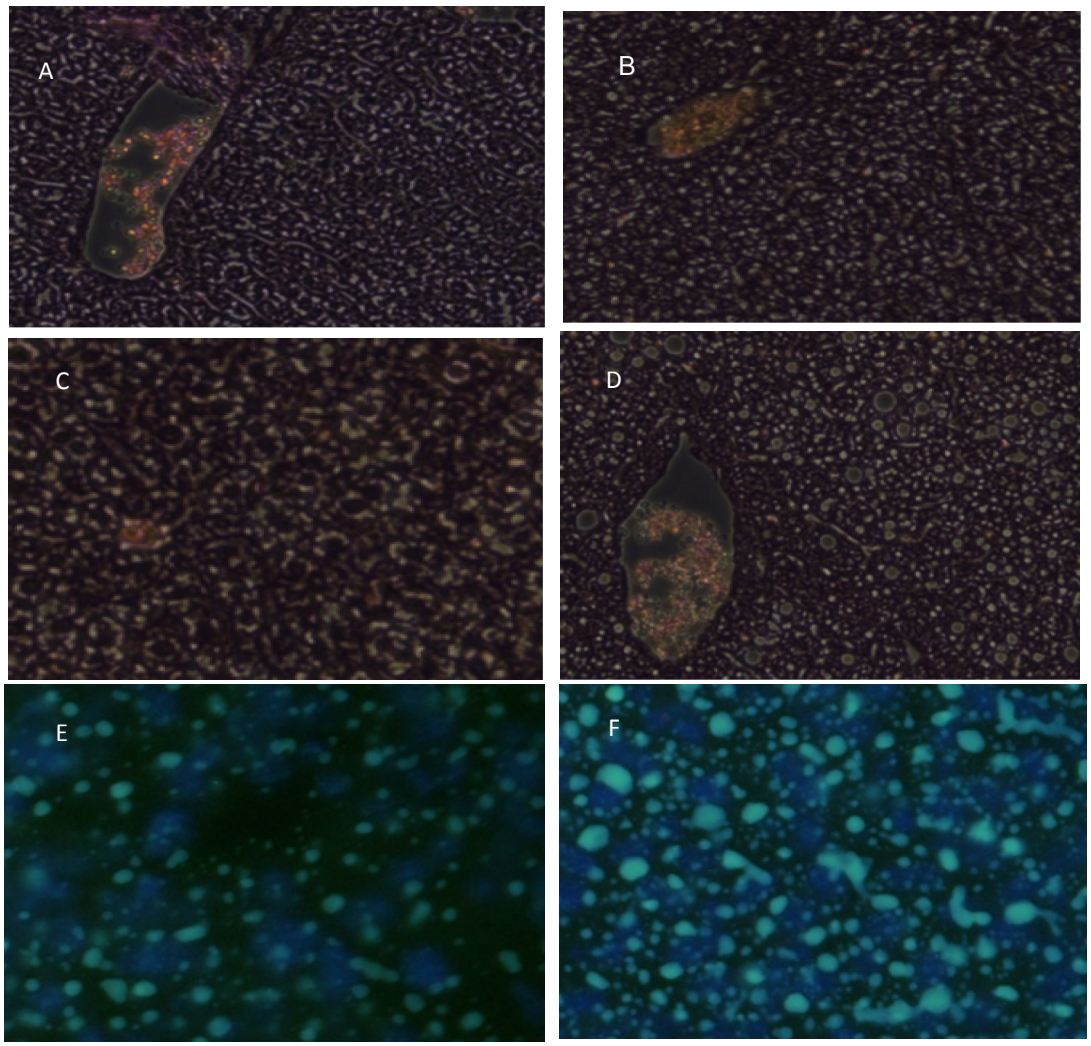

Fig. 2. Liver histology: H&E staining of (A)Chow-control mice; (B) Chow-EtOH mice; (C) LCD-control mice and(D) LCD-EtOH mice. BODIPY staining of (E)LCD- control mice and(F) LCD-EtOH mice

3.2 Oxidative Stress in Livers of NS5A-Tg Mice

For this study, we measured oxidative stress by examining TBARS, 4-HNE adducts and glutathione levels. TBARS were elevated 1.2-fold by ethanol exposure in chow-EtOH mice compared with chow-controls. LCD-Control diet by itself significantly up-regulated (1.8–fold) TBARS levels, and no further effect of ethanol in the liquid diet was observed (Fig. 3A).

There were no ethanol-induced changes in 4-HNE content in mice fed either dietary regimen; however, in LCD- control & EtOH-fed- mice, the amount of 4-HNE was 26% higher compared with chow-EtOH mice (Fig. 3B). GSH was 1.3-fold lower in chow-EtOH mice compared with chow-control, but ethanol exposure reduced GSH levels 3-foldin LCD mice. Ethanol feeding decreased total glutathione by 1.3–fold in Chow-fed mice vs 1.7–fold in LCD mice (Fig. 3C). All these changes were not related to differential expression of NS5A protein in the treatment groups since we observed no difference in NS5A protein levels between control and ethanol-fed mice (data not shown).

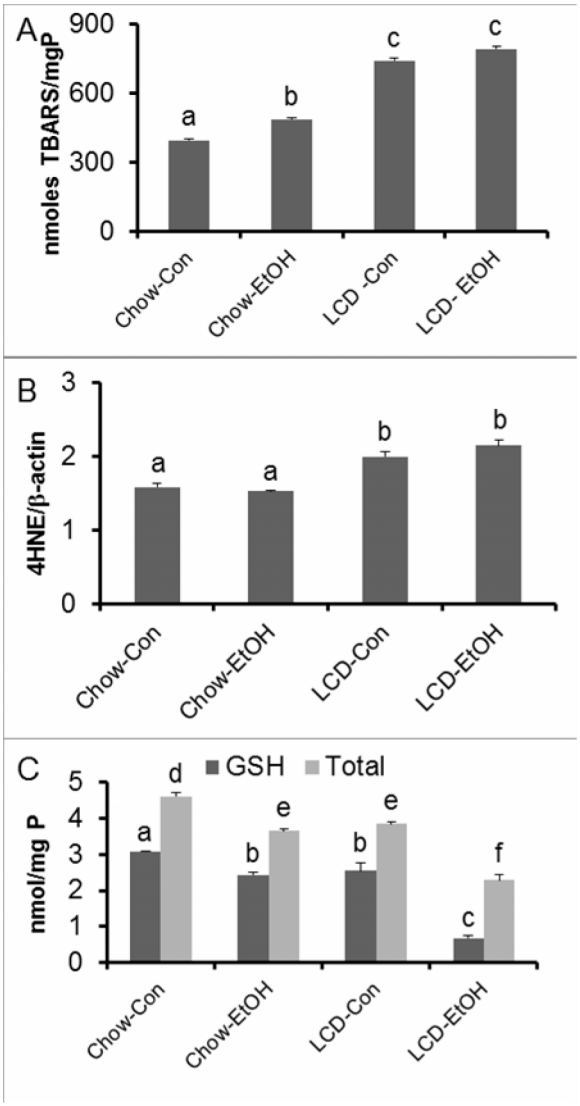

Fig. 3. Indices of oxidative stress in livers of Chow- and LCD-Control and –EtOH mice. (A) TBARS; (B) 4-HNE and \(C)GSH/GSH+GSSG levels were determined as described in Method Section. All data are presented as Mean ±SEM. Values not sharing a common subscript letter are statistically different, P= 0.027 or less

3.3 Cellular Methylation Potential

Ethanol did not affect SAM:SAH ratio when administered in drinking water. However, in LCD mice, ethanol exposure suppressed SAM:SAH ratio by 1.9-fold (Fig. 4).

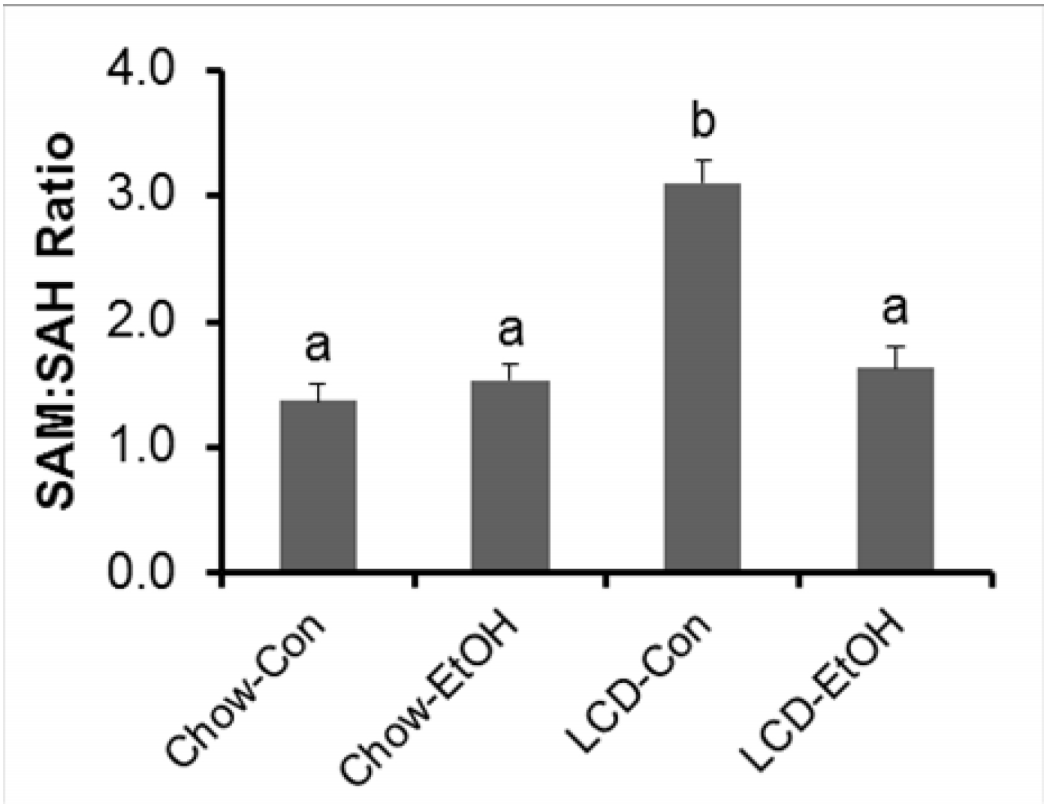

Fig. 4. SAM: SAH ratio in livers of Chow- and LCD-Control and –EtOH mice. SAM and SAH were quantified by HPLC. All data are presented as Mean ± SEM. Values not sharing a common subscript letter are statistically different, P=0.013

3.4 Proteasome Activity: Nuclear vs Cytosolic Fractions

In hepatic nuclear fractions, chow-ethanol feeding had no effect on proteasome activity, but LCD- EtOH feeding decreased nuclear proteasome activity by 25% (Fig.5A). In hepatic cytosolic fractions, there was about 25% reduction in proteasome activity in mice fed by ethanol in both diets (Fig. 5B). However, cytosolic proteasome activity of LCD-control mice was 1.4-fold higher than in the corresponding chow-control mice suggesting that LCD diet by itself enhances cytosolic proteasome function.

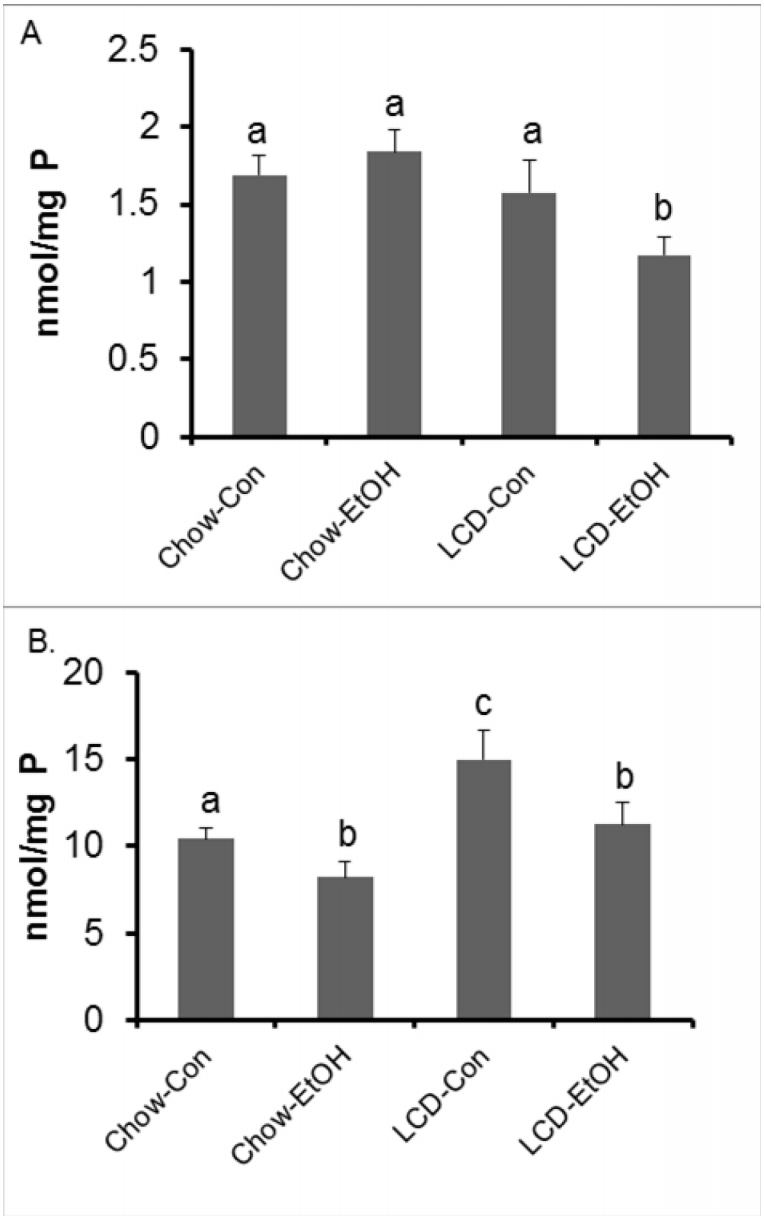

Fig. 5. Proteasome activity in livers of chow- and LCD-control and –EtOH mice. (A) Nuclear proteasome activity and (B) Cytosolic proteasome activity was detected using fluorogenic substrate, SUC-LLVY. All data are presented as Mean ± SEM. Values not sharing a common subscript letter are statistically different, P=0.03 or less

4. DISCUSSION

There are very few studies that compare the mode of ethanol administration in relation to the magnitude of liver injury induced. However, a study previously conducted in rodents showed similar morphological changes in the liver when fed alcohol in liquid diet orin drinking water

[19]. This suggests that the mode of ethanol feeding depends on the investigator's convenience. The present study was conducted to further examine and elucidate the advantages, limitations and differences in liver injury using two modes of chronic ethanol exposure to NS5ATg mice.

By comparing the canonic effects of ethanol on induction of liver pathology, we demonstrated that various types of oral ethanol administration (20% v/v ethanol in water or as 29.2% by calories in LC liquid diet) provided differential effects on livers of NS5A-tg mice. There are several explanations for these events. LCD contains about 35% of fat calories and is considered as a "fat" diet [20]. Thus, unlike Chow + ethanol in water, LCD has a fat-nutritional component. In fact, we found that steatosis (based on H&E staining, Bodipy-stained lipid droplets and triglyceride content was increased in LCD-EtOH, but not in chow-EtOH NS5A mice. These results demonstrate that steatotic changes are induced not by pure ethanol feeding, but by feeding ethanol in a diet rich in unsaturated fats.

Although many factors contribute to liver steatosis development in ethanol-fed mice, we focused on two mechanisms: changes in methylation status (SAM:SAH ratio) and induction of oxidative stress. Interestingly, only LCD-EtOH feeding to NS5A mice led to a lowering of SAM:SAH ratio, that has been previously shown to correlate with fat accumulation and increased liver toxicity [21-23]. Furthermore, various modes of ethanol administration created differential levels of oxidative stress in the liver. Since oxidative stress results from an imbalance between oxidation and anti-oxidative defense, we measured both these factors in order to characterize the level of oxidative stress. Lipid peroxidation products (TBARS and 4-HNE) and a potent anti-oxidant, glutathione in mouse livers were examined. We observed higher TBARS and 4HNE in LCD-EtOH than in chow-EtOH-fed mice. In addition, GSH and total glutathione (GSH+GSSG) were lower in LCD-EtOH mice, suggesting that overall shift to a pro-oxidative state is higher in livers of LCD-EtOH mice compared with chow-EtOH-fed mice, possibly due to decreased anti-oxidant protection. Indeed, if to roughly express oxidative stress as a ratio between TBARS and total GSH, the numbers will be 86, 133, 193 and 347 in Chow-control, Chow-EtOH, LCD-control and LCD-EtOH mice, respectively. Thus, ethanol feeding enhances oxidative stress by about 50% in Chow-fed mice and by 80% in LCD mice over control equal to 100% for each type of diet. Importantly, enhanced lipid peroxidation products in LCD group could not be attributed only to ethanol feeding. Thus, comparatively high TBARS and 4HNE levels were observed in both LCD-control and EtOH mice, indicating that high-fat LCD feeding may induce subsequent protein adduction even in the absence of ethanol. Thus, investigators should be really careful in choosing the mode of ethanol feeding due to enhanced sensitivity of some parameters to lipid peroxidation. As an example, while previously studying how ethanol affects antigen presentation in hepatocytes, we were forced to switch from LCD to Chow diet as LCD-control diet masked the effects of ethanol on the expression of immunoproteasome subunits [14] .

Although NS5A protein by itself can induce oxidative stress [24], it seems unlikely that high level of adducts in LCD mice is due to the expression of HCV protein since we observed no products of lipid peroxidation in NS5A chow control mice despite similar NS5A protein expression under all feeding conditions. Conversely, significantly elevated TBARS were found in only chow-EtOH-fed mice in conjunction with decreased total glutathione. These results indicate that ethanol specific effects were discernible only in chow fed NS5A mice given ethanol in drinking water.

As reveled from our previous studies, proteasome activity is tightly regulated by oxidative stress [18,25-27]. Furthermore, the levels of oxidative stress provide biphasic effects on

proteasome activity: it is induced by low oxidative stress level and is suppressed by high oxidative stress [28]. In this study, we examined proteasome activity as an indirect indicator of the level of oxidative stress and observed that ethanol feeding using two types of dietary regimens suppressed cytosolic proteasome activity. Here, various types of diets caused development of different levels of ethanol-induced oxidative stress. Therefore, the suppression of cytosolic proteasome activity corresponded to the elevation of TBARS and concomitant decreased glutathione in animals fed ethanol by either modes. However, nuclear proteasome activity was decreased only in LCD ethanol-fed mice that developed the highest oxidative stress (with an index of oxidative stress equal to 347 compared with 193 in Chow-EtOH group). These findings indicate that nuclear proteasome is only sensitive to high levels of oxidative stress induced in LCD-EtOH group.

Previously, we have shown that methylation status also contributes to impaired proteasome activity [29]. In Chow-EtOH mice, proteasome activity was also lower than in corresponding control group, but SAM:SAH ratio was not changed indicating that oxidative stress alone can regulate proteasome activity. However, under conditions of both enhanced oxidative and reduced methylation potential as seen in LCD-EtOH-fed mice, a profound impairment in proteasome activity was observed. We cannot exclude the possibility that the accumulation of fat in liver cells due to the reduced SAM:SAH ratio potentiates proteasome dysfunction generated by alcohol-induced oxidative stress. In fact, *in vitro* delivery of oleic acid to Huh7 cells that express CYP2E1 further decreased proteasome activity in ethanol-treated cells (unpublished data), indicating that fat accumulation contributes to suppression of proteasome. Because 20S proteasome is a major intracellular enzyme responsible for degradation of oxidized proteins [30], suppression of proteasome activity may cause feedback up-regulation of oxidatively-modified proteins expression. Indeed, high TBARS levels persisted in ethanol-fed NS5A-Tg-mice that have the lowest proteasome activity. However, the combination of the high oxidative stress and the reduced SAM:SAH ratio in LCD-EtOH mice generated considerable steatosis and increased ALT, suggesting that oxidative stress is not the only pre-requisite factor for subsequent liver injury.

One of the reasons why we used NS5A-Tg mice as a model to study the different modes of ethanol administration is because the expression of HCV non-structural protein, NS5A that is transgenically expressed by liver cells allows investigating the pathogenesis of HCV as well as the potentiating effects of ethanol on liver injury. Currently, most of the data characterizing the synergistic effects of ethanol with the structural HCV protein is focused on examining the effect of core protein in the induction of liver steatosis, oxidative stress and changes in proteasome function [14,28,31,32]. However, NS5A protein is a part of HCV replication complex that sensitizes liver cells to the effects of ethanol [10]. Therefore, it is important to find the right model of oral ethanol feeding that is easily achievable in all laboratories to test a given hypothesis. Learning the advantages and disadvantages of various modes of ethanol feeding of NS5A mice presents opportunities for future investigations on the cross talk between ethanol and non-structural NS5A protein in HCV pathogenesis.

5. CONCLUSION

In conclusion, both modes of oral ethanol feeding (chow and LCD-based) induce oxidative stress in NS5A mice that suppresses proteasome activity. In LCD-EtOH diet, high oxidative stress is accompanied by changes in hepatocellular SAM: SAH levels. However, in control animals, LCD diet (but not chow diet) by itself induces lipid peroxidation, which can in some cases mask the effects of ethanol. Thus, the choice of ethanol feeding modality has to

depend on the endpoints to characterize particular biochemical parameters of ethanol metabolisms in the liver.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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