

1 Mode of Oral Ethanol Feeding Affects Liver Oxidative Stress

2 Levels And Methylation

3 **Background:** Ethanol affects the progression and outcomes of hepatitis C viral (HCV)
 4 infection and various organs and systems in heavy and moderate drinkers. Our aim
 5 was to investigate the effects of two modes of oral ethanol feeding on induction of
 6 oxidative stress, impaired methylation status and downstream changes in proteasome
 7 activity in livers of NS5A-transgenic (Tg) mice.**Methods:** We compared two modes of
 8 oral ethanol feeding in which ethanol was either given in water (chow fed mice given
 9 20% ethanol in water; designated chow-EtOH) or fed in Lieber De Carli liquid diet (LCD-
 10 EtOH). Appropriate controls were used. The mechanisms of alcohol and NS5A-induced
 11 changes in oxidative stress and liver methylation status and downstream 20S
 12 proteasome activity were determined. **Results:** We found that ethanol administration
 13 using both feeding regimens induced oxidative stress and suppressed cytosolic
 14 proteasome activity. It appeared that only LCD-EtOH diet induced fatty changes in the
 15 liver, which corresponded to higher levels of oxidative stress, methylation status
 16 impairment and reduction of both cytosolic and nuclear proteasome activity. However,
 17 LCD diet by itself triggered lipid peroxidation. We **conclude** that in NS5A-Tg mice, both
 18 modes of oral ethanol feeding (chow and LCD-based) induce oxidative stress that
 19 suppresses proteasome activity. Nonetheless, methylation impairment and higher level
 20 of this stress as well as suppression of nuclear proteasome was observed in LCD-EtOH
 21 mice. However, because of high sensitivity of NS5A-Tg mice to LCD, the effects of
 22 control liquid diet on lipid peroxidation in certain cases mask the effects of ethanol.

23 **Key words:** NS5A mice, ethanol feeding, oxidative stress, methylation status,
24 proteasome activity

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

30 1. INTRODUCTION

31 Alcohol damages various organs and systems in heavy and moderate drinkers. Since
32 the liver is the major site of ethanol metabolism, alcoholic liver disease (ALD) is a
33 frequently observed in alcoholics. It is accepted that the effects of ethanol on liver
34 pathology is due to its metabolism and dysregulation of multiple signal transduction
35 pathways that subsequently impairs liver cell function [1-6]. *In vivo* consequences of
36 ethanol metabolism are studied on small rodent models. These models usually do not
37 exhibit the whole spectrum of changes typical for alcohol-consuming humans, but there
38 are still certain pathological features that can be induced by ethanol feeding. The
39 expression of these features depends on multiple factors: the strain of mice, their
40 genotypic characteristics (knockouts), nutritional factors, supplements and the mode of
41 alcohol feeding. Here, we compared two modes of chronic ethanol feeding: ethanol in
42 liquid LieberDeCarli Diet or in drinking water, to characterize the level of ethanol-induced
43 changes in liver cells, such as necrotic cell death (alanine aminotransferases, ALT),
44 steatosis, oxidative stress and alterations in methylation status. In addition, to monitor
45 the parameter that is downstream from oxidative stress and is partially dependent on

methylation status (the intracellular ratio of S-adenosylmethionine and S-adenosylhomocysteine, SAM:SAH), we measured liver proteasome activity. All these changes are the most common features of alcohol-induced liver damage, and this study is designed to characterize the advantages and disadvantages of various modes of ethanol feeding to mice that potentially can be used for further investigations of 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. 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 [7]. We therefore expected NS5A mice to be highly sensitive to ethanol treatment and generate a clinically relevant model to conduct comparative studies using various ethanol feeding modes in the development of liver pathology.

2. MATERIALS AND METHODS

2.1. Mice: We used transgenic FVB mice, which express NS5A (non-structural HCV) protein (NS5A-tg mice) and were matched in age and weight. Mice were obtained from Dr. Ratna Ray, Saint Louis University. The details about these mice were published elsewhere [8]. 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. An 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. Mice were treated in accordance with criteria outlined in the “Guide for the Care and Use of Laboratory Animals”.

2.2. Ethanol feeding: Ethanol was administered either in water (20% ethanol water, Chow-EtOH) [9] or as part of the LieberDeCarli liquid diet with 29.2% of calories derived from ethanol (LCD-EtOH) as previously described [10]. The control mice for these two ethanol groups were given either water alone plus LabDiets 5001 Purina chow (Chow-Control)[9, 11] or pair-fed the LieberDeCarli 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 -80°C 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 4°C to yield the cytosol fractions (supernatants). (C) **Nuclear fractions** were obtained from livers according the method of Andrews[12].

2.4. Oxidative stress-related parameters were examined in total liver homogenates.- Oxidant formation was documented 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 (Ab from Cell Biolabs, Inc). Antioxidant defense was quantified by measuring glutathione levels using the enzymatic recycling method[13].

2.5. Cellular methylation potential was measured by determining hepatic SAM and SAH levels by HPLC analysis [14].

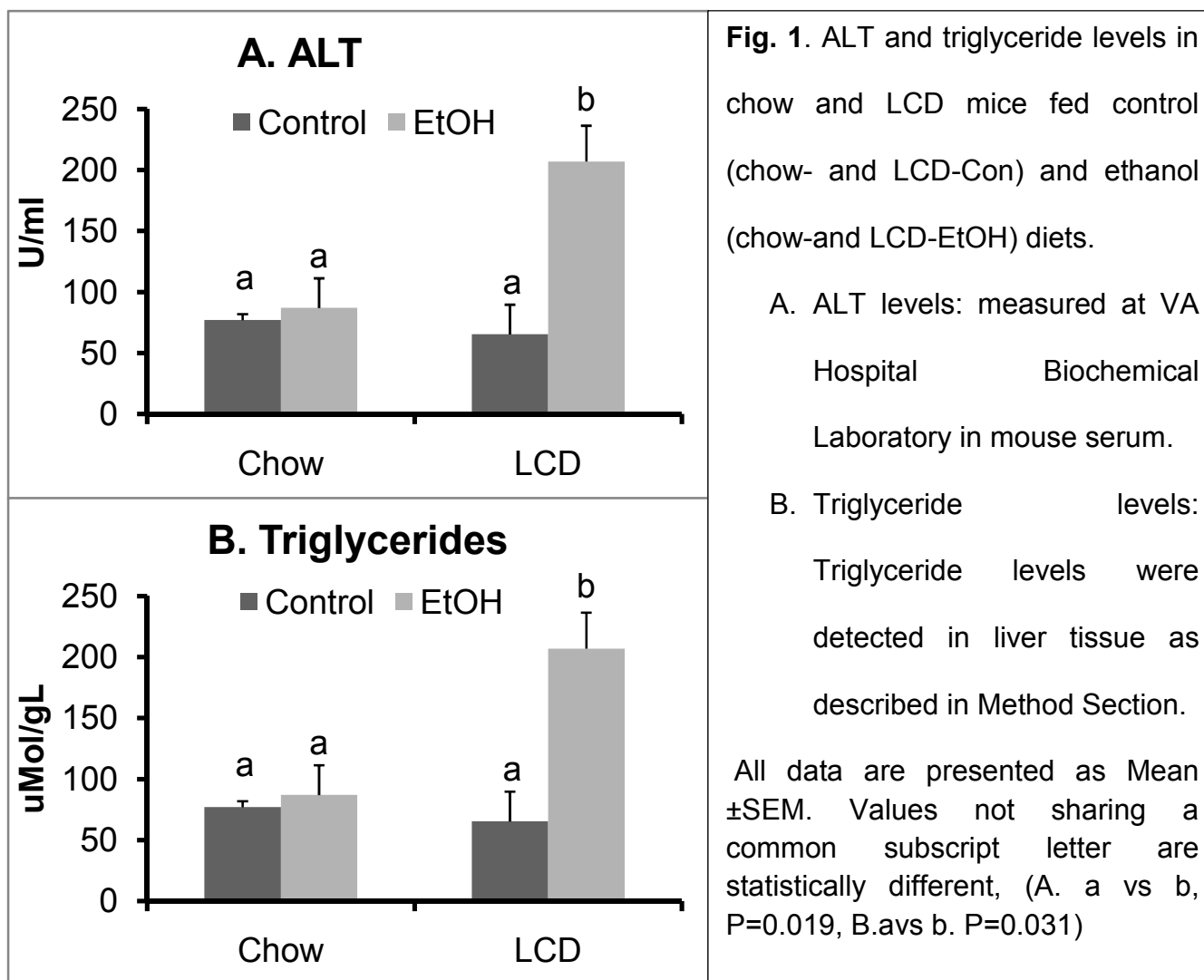
2.6. Lipid accumulation in the liver was measured by determining triglyceride levels [14]and H&E staining. Cytoplasmic lipid droplets (LDs) were visualized by BODIPY staining of fresh frozen (OCT) liver sections.

2.7. 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 [15].

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). We observed no difference in NS5A protein expression between control and ethanol-fed mice (not shown).

2.9. Statistical analyses: Data are expressed as mean values $\pm$ SEM. Comparisons among multiple groups were determined by one-way ANOVA, using a Tukey post-hoc test. For comparisons between two groups, we usedStudent'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 levels were unchanged in chow-EtOH-fed mice compared to their chow-fed controls. However, there was a threefold rise in ALT level in LCD-EtOH mice compared with pair-fed LCD-Control mice (Fig.1A).

3.1.2. Steatosis: There were no differences in triglyceride levels between chow-control-fed and chow-ETOH-fed groups. However, the level of triglycerides was observed in mice fed LCD- Control diet compared with Chow-Control mice was a 2-fold

119

120

121

122

123

124

125

126

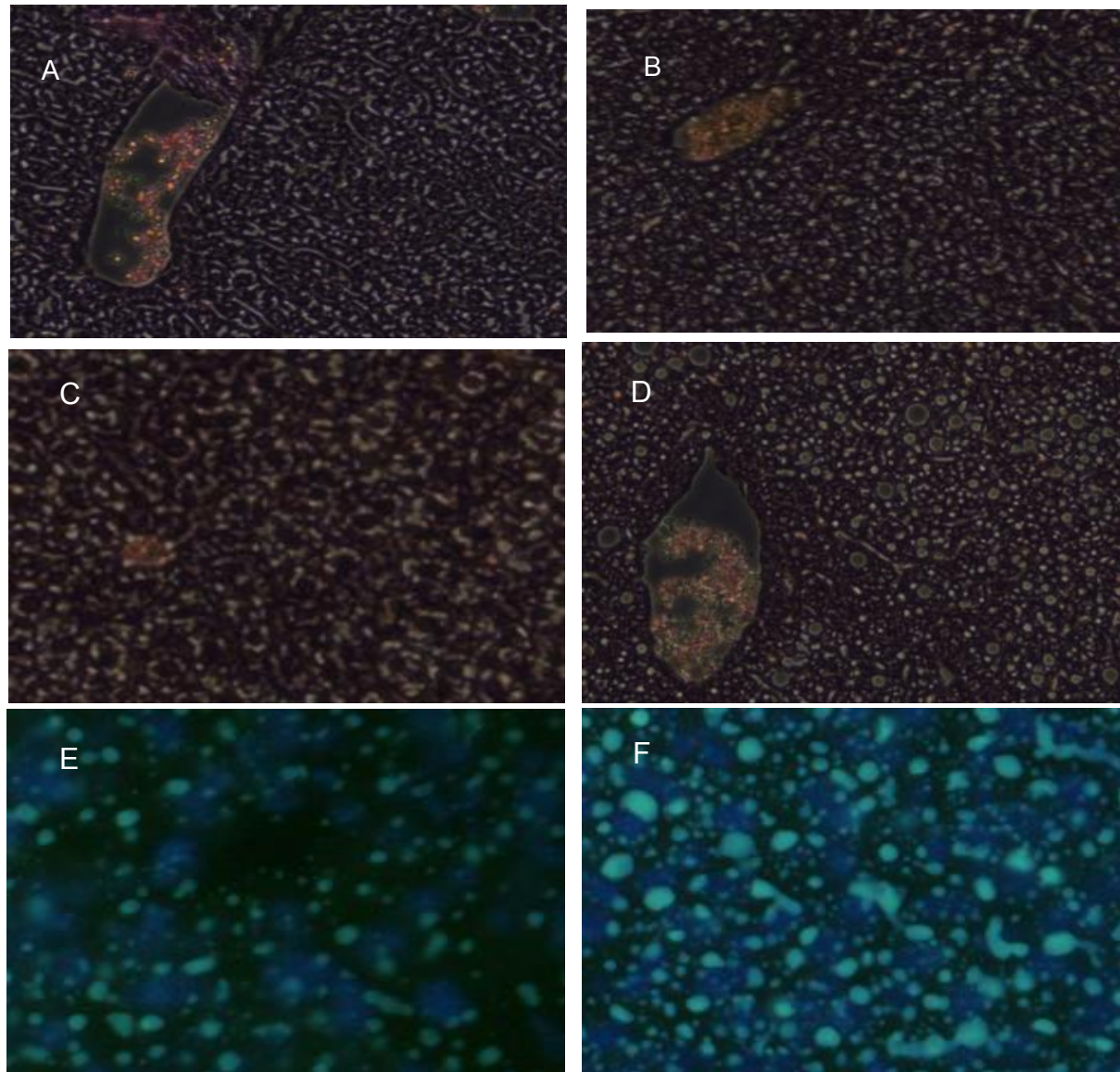
127

128

129

130

131



132

133

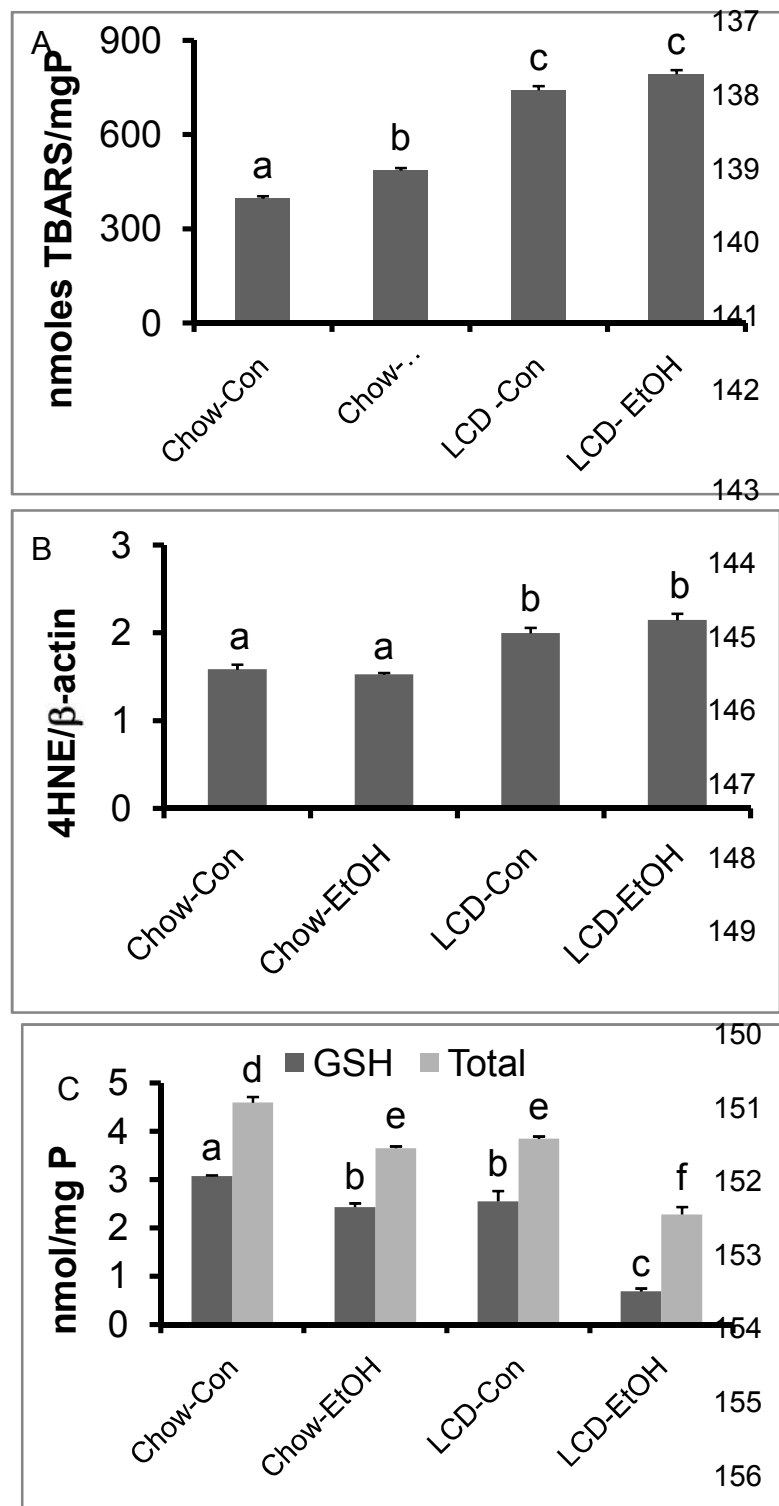
Fig.2. Liver histology: A. H&E staining, Chow-control mice; B. H&E staining, Chow-EtOH mice; C. H&E staining, LCD-control mice; D. H&E staining, LCD-EtOH mice; E. BODIPY staining, LCD- control mice; F. BODIPY staining, LCD-EtOH

134

135

136

higher. LCD ethanol feeding to mice 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, (Fig 2 A, B, C, D)).

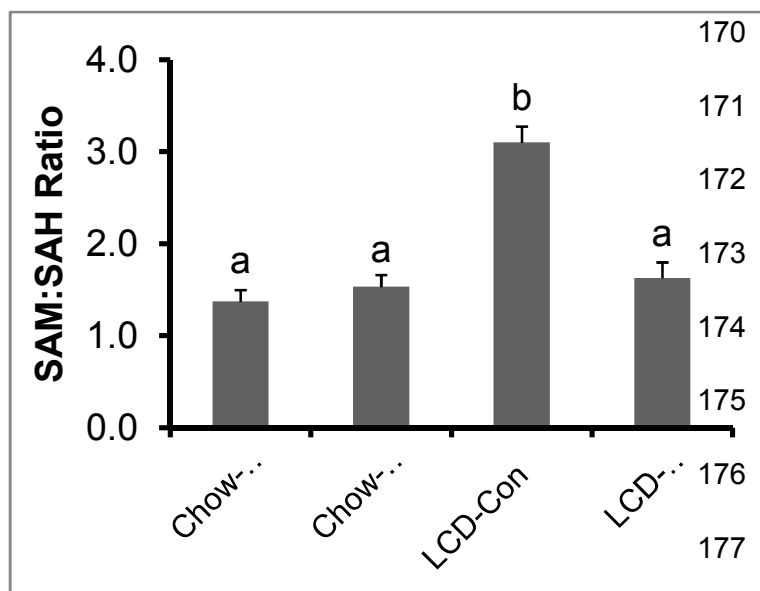
Since ethanol administration in water revealed no changes in the liver steatosis assessed biochemically (triglyceride levels) or by histological evaluation (H&E staining), we performed BODIPY staining

only in LCD-fed mice (Fig. 2E and F). It appeared that there was visual increase in number of LDs between control and EtOH-fed NS5A-Tg mice, as well as the size of some LDs that was larger in EtOH-fed

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

159 mice; however, LDs were also detected in the LCD-Control group.

160 **3.2. Oxidative stress in livers of NS5A-Tg mice:** For this study, we measured
 161 oxidative stress by examining TBARS, 4-HNE adducts and glutathione levels. TBARS
 162 were elevated 1.2-fold by ethanol exposure in chow-EtOH mice; LCD-Control diet by
 163 itself significantly up-regulated (1.8-fold) TBARS levels, and no further effect of ethanol
 164 in liquid diet was seen (Fig. 3A). There were no ethanol-induced changes in 4-HNE
 165 content in mice fed either dietary regimen; however, in LCD- control &EtOH-fed- mice,
 166 the amount of 4-HNE was 26% higher compared with chow-EtOH mice (Fig. 3B). GSH
 167 was 1.3-fold lower in chow-EtOH mice compared with chow-control, but in LCD mice,
 168 ethanol exposure reduced GSH levels 3-fold. Ethanol feeding decreased total
 169 glutathione by 1.3-fold in Chow-fed mice vs 1.7-fold in LCD mice (Fig. 3C).



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 1.9-fold (Fig. 4A).

178

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.

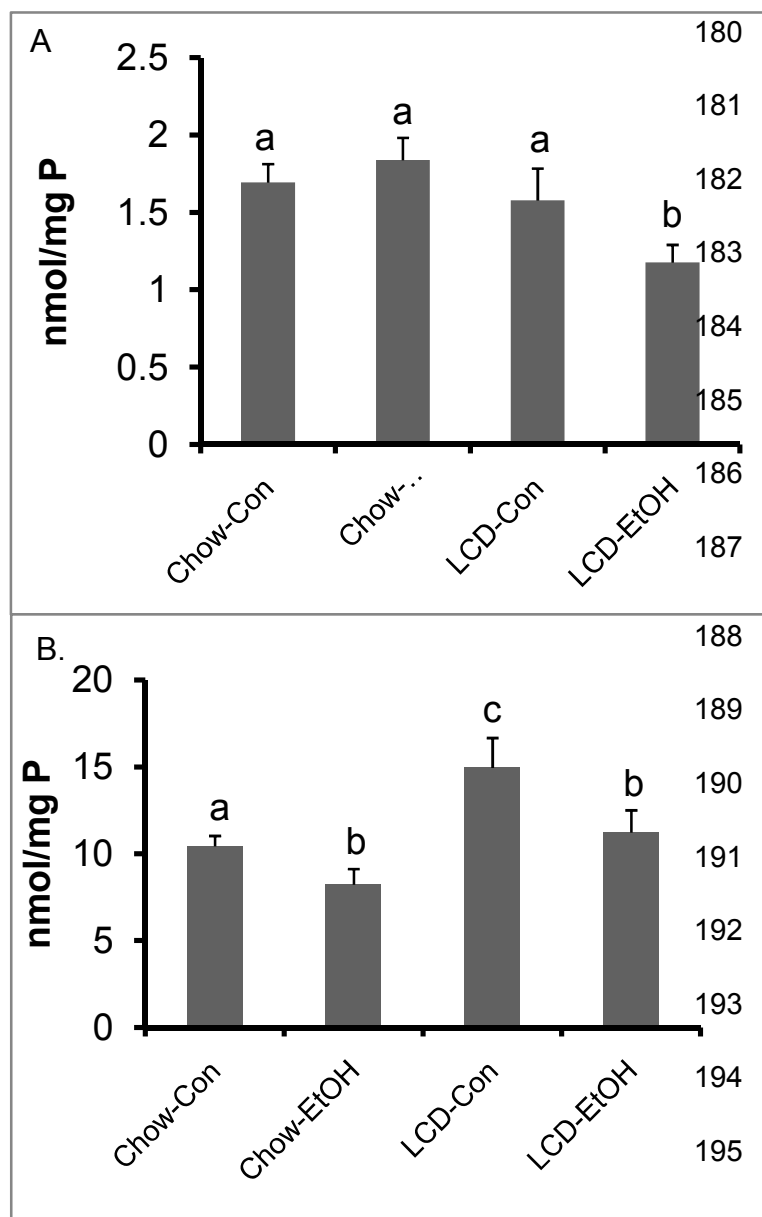


Fig. 5. Proteasome activity in livers of chow- and LCD-control and -EtOH mice. A. Nuclear proteasome activity. B. Cytosolic proteasome activity. Proteasome activity was detected using fluorogenic substrate, Suc-LLVY. All data are presented as Mean $\pm$ SEM. Values not sharing a common subscript letter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

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 corresponding chow-

control mice suggesting that

LCD diet by itself enhances cytosolic proteasome function.

200

201 **4. DISCUSSION:**

202 There are limited numbers of studies that compare the mode of ethanol administration
 203 on the extent of liver injury. However, a study previously conducted in rodents showed
 204 similar morphological changes in the liver when fed alcohol in liquid diet or in drinking
 205 water [16]. This suggests that the mode of ethanol feeding depends on the
 206 investigator's convenience. The present study was conducted to further examine and
 207 elucidate the advantages, limitations and differences in liver injury using two different
 208 modes of chronic ethanol exposure to NS5ATg mice.

209 By comparing the canonical effects of ethanol on induction of liver pathology, we
 210 demonstrated that various types of oral ethanol administration (20% v/v ethanol in water
 211 or as 29.2% by calories in LC liquid diet) provided differential effects on livers of NS5A-
 212 tg mice. There are several explanations for these events. LCD contains about 35% of
 213 fat calories and is considered as a "fat" diet [17]. Thus, unlike Chow + ethanol in water,
 214 LCD has a fat-nutritional component. In fact, we found that steatosis (based on H&E
 215 staining, Bodipy-stained lipid droplets and triglyceride content) was increased in LCD-
 216 EtOH, but not in chow-EtOH NS5A mice. These results demonstrate that steatotic
 217 changes are induced not by pure ethanol feeding, but by feeding ethanol in a diet rich in
 218 unsaturated fat.

219 Although many factors contribute to liver steatosis development in ethanol-fed mice, we
 220 focused on two mechanisms: changes in methylation status (SAM:SAH ratio) and
 221 induction of oxidative stress. Interestingly, only LCD-EtOH feeding to NS5A mice led to
 222 a lowering of SAM:SAH ratio, that has been previously shown to correlate with fat

223 accumulation and increased liver toxicity [18-20]. Furthermore, various modes of ethanol
 224 administration created differential levels of oxidative stress in the liver. Since oxidative
 225 stress results from an imbalance between oxidation and anti-oxidative defense, we
 226 measured both these factors in order to characterize the level of oxidative stress. Lipid
 227 peroxidation products (TBARS and 4-HNE) and a potent anti-oxidant, glutathione in
 228 mouse livers were examined. We observed higher TBARS and 4HNE in LCD-EtOH
 229 than in chow-EtOH-fed mice. In addition, GSH and total glutathione (GSH+GSSG) were
 230 lower in LCD-EtOH mice, suggesting that overall shift to a pro-oxidative state is higher
 231 in livers of LCD-EtOH mice compared with chow-EtOH-fed mice, possibly due to
 232 decreased anti-oxidant protection. Indeed, if to roughly express oxidative stress as a ratio
 233 between TBARS and total GSH, the numbers will be 86, 133, 193 and 347 in Chow-
 234 control, Chow-EtOH, LCD-control and LCD-EtOH mice, correspondingly. Thus, ethanol
 235 feeding enhances oxidative stress by about 50% in Chow-fed mice and by 80% in LCD
 236 mice over control equal to 100% for each type of diet. Importantly, enhanced lipid
 237 peroxidation products in LCD group could not be attributed only to ethanol feeding.
 238 Thus, comparatively high TBARS and 4HNE levels were observed in both LCD-control
 239 and EtOH mice, indicating that high-fat LCD feeding may induce subsequent protein
 240 adduction even in the absence of ethanol. Thus, investigators should be really careful in
 241 choosing the mode of ethanol feeding due to enhanced sensitivity of some parameters
 242 to lipid peroxidation. As an example, previously, while studying how ethanol affects
 243 antigen presentation in hepatocytes, we were forced to switch from LCD to Chow diet
 244 since LCD-control diet masked the effects of ethanol on immunoproteasome subunits
 245 expression [11, 15].

246 Although NS5A protein by itself can induce oxidative stress[21], it seems unlikely that
 247 high level of adducts in LCD mice is due to the expression of HCV protein since we
 248 observed no products of lipid peroxidation in NS5A chow control mice despite similar
 249 NS5A protein expression under all feeding conditions. Conversely, significantly elevated
 250 TBARS were found in only chow-EtOH-fed mice in conjunction with decreased total
 251 glutathione. These results indicate that ethanol specific effects were discernible only in
 252 chow fed NS5A mice given ethanol in drinking water.

253 As revealed from our previous studies, proteasome activity is tightly regulated by
 254 oxidative stress [15, 22-24]. Furthermore, the levels of oxidative stress provide biphasic
 255 effects on proteasome activity: it is induced by low oxidative stress level and is
 256 suppressed by high oxidative stress[25]. In this study, we examined proteasome activity
 257 as an indirect indicator of the level of oxidative stress and observed that ethanol feeding
 258 using two types of dietary regimens suppressed cytosolic proteasome activity. Here,
 259 various types of diets caused development of different levels of ethanol-induced
 260 oxidative stress. Therefore, the suppression of cytosolic proteasome activity
 261 corresponded to the elevation of TBARS and concomitant decreased glutathione in
 262 animals fed ethanol by either modes. However, nuclear proteasome activity was
 263 decreased only in LCD ethanol-fed mice that developed the highest oxidative stress (with
 264 an index of oxidative stress equal to 347 compared with 193 in Chow-EtOH group).
 265 These findings indicate that nuclear proteasome is only sensitive to high levels of
 266 oxidative stress induced in LCD-EtOH group.

267 Previously, we have shown that methylation status also contributes to impaired
 268 proteasome activity [26]. 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 happens in LCD-EtOH, 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[27], suppression of proteasome activity may cause feedback up-regulation of oxidatively-modified protein 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 for 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 HCV pathogenesis as well as the potentiating effect of ethanol on these mice. 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 [11, 25, 28, 29].

292 However, NS5A protein is a part of HCV replication complex that sensitizes liver cells to
 293 the effects of ethanol[7]. Therefore, it is important to find the right model of oral ethanol
 294 feeding that is easily achievable in most all laboratories to test a given hypothesis.
 295 Learning the advantages and disadvantages of various modes of ethanol feeding of
 296 NS5A mice presents opportunities for future investigations on the cross talk between
 297 ethanol and non-structural NS5A protein in HCV pathogenesis.

298 **In conclusion**, both modes of oral ethanol feeding (chow and LCD-based) induce
 299 oxidative stress in NS5A mice that suppresses proteasome activity. In LCD-EtOH diet,
 300 high oxidative stress is accompanied by changes in SAM: SAH levels. However, in
 301 control animals, LCD diet (but not chow diet) by itself induces lipid peroxidation, which
 302 can partially mask the effects of ethanol. Thus, the choice of ethanol feeding modality
 303 has to depend on the endpoints to characterize particular biochemical parameters of
 304 ethanol metabolisms in the liver.

305 **Competing interests:**

306 Authors have declared that no competing interests exist.

307 **REFERENCES:**

- 308 1. Osna N, Donohue, T, White, R, Beard, M, Kharbanda, K. Ethanol and hepatitis C
 309 viral proteins regulate interferon signaling in liver cells via impaired methylation of
 310 STAT1. *Hepatology* 2008; **48**: 75A.
- 311 2. Osna NA, White RL, Thiele GM, Donohue TM, Jr. Ethanol metabolism alters
 312 major histocompatibility complex class I-restricted antigen presentation in liver
 313 cells. *Hepatology* 2009; **49**: 1308-15.

- 314 3. Donohue TM, Osna NA, Clemens DL. Recombinant Hep G2 cells that express
315 alcohol dehydrogenase and cytochrome P450 2E1 as a model of ethanol-elicited
316 cytotoxicity. *Int J Biochem Cell Biol* 2006; **38**: 92-101.
- 317 4. Cederbaum AI. Role of CYP2E1 in ethanol-induced oxidant stress, fatty liver and
318 hepatotoxicity. *Dig Dis* 2010; **28**: 802-11.
- 319 5. Bardag-Gorce F, Li J, French BA, French SW. The effect of ethanol-induced
320 CYP2E1 on proteasome activity: the role of 4-hydroxynonenal. *Exp Mol Pathol*
321 2005; **78**: 109-15.
- 322 6. Thomes PG, Osna NA, Davis JS, Donohue TM, Jr. Cellular steatosis in ethanol
323 oxidizing-HepG2 cells is partially controlled by the transcription factor, early
324 growth response-1. *Int J Biochem Cell Biol* 2013; **45**: 454-63.
- 325 7. Machida K, Tsukamoto H, Mkrtchyan H et al. Toll-like receptor 4 mediates
326 synergism between alcohol and HCV in hepatic oncogenesis involving stem cell
327 marker Nanog. *Proc Natl Acad Sci U S A* 2009; **106**: 1548-53.
- 328 8. Kanda T, Steele R, Ray R, Ray RB. Inhibition of intrahepatic gamma interferon
329 production by hepatitis C virus nonstructural protein 5A in transgenic mice. *J Virol*
330 2009; **83**: 8463-9.
- 331 9. Song K, Coleman RA, Zhu X et al. Chronic ethanol consumption by mice results
332 in activated splenic T cells. *J Leukoc Biol* 2002; **72**: 1109-16.
- 333 10. Donohue TM, Jr., Curry-McCoy TV, Todero SL et al. L-Buthionine (S,R)
334 sulfoximine depletes hepatic glutathione but protects against ethanol-induced
335 liver injury. *Alcohol Clin Exp Res* 2007; **31**: 1053-60.

- 336 11. Osna NA, Bardag-Gorce F, White RL et al. Ethanol and hepatitis C virus
337 suppress peptide-MHC class I presentation in hepatocytes by altering
338 proteasome function. *Alcohol Clin Exp Res* 2012; **36**: 2028-35.
- 339 12. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of
340 DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids*
341 *Res* 1991; **19**: 2499.
- 342 13. Tietze F. Enzymic method for quantitative determination of nanogram amounts of
343 total and oxidized glutathione: applications to mammalian blood and other
344 tissues. *Anal Biochem* 1969; **27**: 502-22.
- 345 14. Kharbanda KK. Role of transmethylation reactions in alcoholic liver disease.
346 *World J Gastroenterol* 2007; **13**: 4947-54.
- 347 15. Osna NA, White RL, Todero S et al. Ethanol-induced oxidative stress suppresses
348 generation of peptides for antigen presentation by hepatoma cells. *Hepatology*
349 2007; **45**: 53-61.
- 350 16. Sarphie TG, D'Souza NB, Spitzer JJ, Deaciuc IV. Chronic alcohol feeding in
351 liquid diet or in drinking water has similar effects on electron microscopic
352 appearance of the hepatic sinusoid in the rat. *Alcohol Clin Exp Res* 1996; **20**:
353 973-9.
- 354 17. Lieber CS. Alcoholic fatty liver: its pathogenesis and mechanism of progression
355 to inflammation and fibrosis. *Alcohol* 2004; **34**: 9-19.
- 356 18. Kharbanda KK, Rogers DD, 2nd, Mailliard ME et al. A comparison of the effects
357 of betaine and S-adenosylmethionine on ethanol-induced changes in methionine
358 metabolism and steatosis in rat hepatocytes. *J Nutr* 2005; **135**: 519-24.

- 359 19. Kharbanda KK, Rogers DD, 2nd, Mailliard ME et al. Role of elevated S-
360 adenosylhomocysteine in rat hepatocyte apoptosis: protection by betaine.
361 *Biochem Pharmacol* 2005; **70**: 1883-90.
- 362 20. Kharbanda KK, Mailliard ME, Baldwin CR et al. Betaine attenuates alcoholic
363 steatosis by restoring phosphatidylcholine generation via the
364 phosphatidylethanolamine methyltransferase pathway. *J Hepatol* 2007; **46**: 314-
365 21.
- 366 21. Wang T, Weinman SA. Interactions Between Hepatitis C Virus and Mitochondria:
367 Impact on Pathogenesis and Innate Immunity. *Curr Pathobiol Rep* 2013; **1**: 179-
368 87.
- 369 22. Osna NA, Haorah J, Krutik VM, Donohue TM, Jr. Peroxynitrite alters the catalytic
370 activity of rodent liver proteasome in vitro and in vivo. *Hepatology* 2004; **40**: 574-
371 82.
- 372 23. Donohue TM, Jr., Kharbanda KK, Casey CA, Nanji AA. Decreased proteasome
373 activity is associated with increased severity of liver pathology and oxidative
374 stress in experimental alcoholic liver disease. *Alcohol Clin Exp Res* 2004; **28**:
375 1257-63.
- 376 24. Donohue TM, Jr., Cederbaum AI, French SW et al. Role of the proteasome in
377 ethanol-induced liver pathology. *Alcohol Clin Exp Res* 2007; **31**: 1446-59.
- 378 25. Osna NA, White RL, Krutik VM et al. Proteasome activation by hepatitis C core
379 protein is reversed by ethanol-induced oxidative stress. *Gastroenterology* 2008;
380 **134**: 2144-52.

- 381 26. Osna NA, White RL, Donohue TM, Jr. et al. Impaired methylation as a novel
382 mechanism for proteasome suppression in liver cells. *Biochem Biophys Res*
383 *Commun* 2010; **391**: 1291-6.
- 384 27. Jung T, Grune T. The proteasome and the degradation of oxidized proteins: Part
385 I-structure of proteasomes. *Redox Biol.* 2013/09/12 ed 2013:178-82.
- 386 28. Wang AG, Lee DS, Moon HB et al. Non-structural 5A protein of hepatitis C virus
387 induces a range of liver pathology in transgenic mice. *J Pathol* 2009; **219**: 253-
388 62.
- 389 29. Ogawa K, Hishiki T, Shimizu Y et al. Hepatitis C virus utilizes lipid droplet for
390 production of infectious virus. *Proc Jpn Acad Ser B Phys Biol Sci* 2009; **85**: 217-
391 28.

392

393

394