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The pathogenesis of ethanol versus methionine and choline deficient diet-induced liver injury

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ABSTRACT

The differences and similarities of the pathogenesis of alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) were examined. Mice (six/group) received one of four Lieber-Decarli liquid diets for 6 weeks: (1) paired-fed control diet; (2) control diet with ethanol (ethanol); (3) paired-fed methionine/choline deficient (MCD) diet; and (4) MCD plus ethanol (combination). Hepatotoxicity, histology, and gene expression changes were examined. Both MCD and ethanol induced macrovesicular steatosis. However, the combination diet produced massive steatosis with minor necrosis and inflammation. MCD and combination diets, but not ethanol, induced serum ALT levels by 1.6- and 10-fold, respectively. MCD diet, but not ethanol, also induced serum alkaline phosphatase levels suggesting bile duct injury. Ethanol increased liver fatty acid binding protein (L-FABP) mRNA and protein levels. In contrast, the combination diet decreased L-FABP mRNA and protein levels and increased hepatic free fatty acid and lipid peroxide levels. Ethanol, but not MCD, reduced hepatic S-adenosylmethionine (SAM) and GSH levels. Hepatic TNFα protein levels were increased in all treatment groups, however, IL-6, a hepatoprotective cytokine which promotes liver regeneration was increased in ethanol-fed mice (2-fold), but decreased in the combination diet-treated mice. In addition, the combination diet reduced phosphorylated STAT3 and Bcl-2 levels. While MCD diet might cause bile duct injury and cholestasis, ethanol preferentially interferes with the SAM-GSH oxidative stress pathway. The exacerbated liver injury induced by the combination diet might be explained by reduced L-FABP, increased free fatty acids, oxidative stress, and decreased IL-6 protein levels. The combination diet is an efficient model of steatohepatitis.

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Abbreviations: L-FABP, liver fatty acid binding protein; GST, glutathione S-transferase; CYP2E1, cytochrome P450 2E1; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; MCD, methionine and choline deficient; ROS, reactive oxygen species; PPARα, peroxisome proliferator-activated receptor alpha; TNFα, tumor necrosis factor-alpha; ASH, alcoholic steatohepatitis; NASH, non-alcoholic steatohepatitis; RT-PCR, reverse transcription-polymerase chain reaction; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LPO, lipid peroxidation; GSH, reduced glutathione; GPX, glutathione peroxidase; NEFA, nonesterified fatty acid; CDNB, 1-chloro-2,4-dinitrobenzene; TBST, Tris-buffered saline with 0.1% Tween 20; MMLV-RT, Molony murine leukemia virus reverse transcriptase; MAT, methionine adenosyltransferase; AOX, acyl-CoA oxidase; STAT3, signal transducer and activator of transcription factor 3; PC, phosphatidylcholine; VLDL, very low density lipoproteins.

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1. Introduction

The cellular and molecular mechanisms involved in the pathogenesis of both alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) are not fully understood [1,2]. However, given their histological similarity, including macrovesicular steatosis, hepatocyte ballooning, necrosis, and inflammation, it is believed that the pathogenesis of ASH and NASH have much in common [2]. The similarities in the pathogenesis between ASH and NASH include the role of oxidative stress, iron deposition, hepatic induction of cytochrome P450 2E1 (Cyp2e1), involvement of endotoxin and/or tumor necrosis factor-alpha (TNFα), and beneficial responses to antioxidant and anti-inflammatory therapies [3–6].

Both chronic ethanol ingestion and obesity are known to stimulate lipogenesis through inhibition of the expression of the peroxisome proliferator-activated receptor-α (PPARα), a nuclear receptor for free fatty acids and crucial for metabolism, oxidation, uptake, and transport of hepatic fatty acids [7,8]. Conversely, the expression of PPARγ, a key regulator of adipocyte differentiation and lipid storage is reported to be increased in the liver during ASH and NASH [9]. Furthermore, during chronic ethanol ingestion, the relative accumulation of NADH resulting from an increased ratio of NADH/NAD^+ stimulates the activity of lipogenic enzymes and hence fatty acid and triglyceride synthesis [10]. In line with its importance, it has been demonstrated that activation of PPARα improves abnormal lipid metabolism, while activation of PPARγ worsens it [11–13].

Recent reports indicate that patients with a fatty liver are more susceptible to liver cirrhosis and hepatocellular carcinoma suggesting that a fatty liver is not considered to be benign [14]. As a result, attention has therefore shifted from the causes of steatosis to understanding the biochemical and pathologic changes underlying the progression of steatosis to steatohepatitis. Insights into these mechanisms are of utmost importance because currently there are no approved pharmacological agents for the treatment of ASH and NASH. In addition to the role of proinflammatory cytokines such as TNFα in ASH and NASH, peroxidative injury through oxidative stress has been suggested as important in the development of steatohepatitis [3–6]. Furthermore, emerging data indicate that hepatocyte apoptosis, a specific form of cell death, may be a key mechanism in the progression of steatosis to steatohepatitis [15].

Both experimental and epidemiological studies have demonstrated that chronic ethanol ingestion causes depletion of nutrients such as choline, folate, and methionine due to malnutrition or toxic interaction of ethanol with their metabolism [16,17]. Diets devoid of methionine and choline provides a standard nutritional rodent model for NASH that produces the key features of human NASH [11,18]. Using the methionine and choline deficient (MCD) diet, it was reported that steatohepatitis induced by this diet is the result of increased generation of reactive oxygen species by both Cyp2e1 and Cyp4a enzymes [11]. Studies examining the potential interaction between ethanol and MCD diet have not been fully addressed. Although ASH and NASH have some similarities, their clinical and laboratory profiles differ, suggesting both shared and unique pathologic mechanisms. For example, while elevations of the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ratio and of γ-glutamyl transpeptidase are relatively characteristic of ASH, such elevations may be mild in NASH [19].

In this study, we compared biochemical and pathologic changes produced by feeding ethanol or MCD diets to examine similarities and potential differences between ASH and NASH. We also investigated the interaction between ASH and NASH by feeding mice with a combination of ethanol and MCD diet. Ethanol ingestion was associated with perturbations in the antioxidant signaling pathway without hepatocyte injury suggesting the involvement of oxidative stress as a major factor in ASH. Hepatic TNFα protein levels were increased in all treatment groups, however, IL-6, a hepatoprotective cytokine which promotes hepatocyte proliferation was increased in ethanol-fed mice. While the MCD diet did not decrease S-adenosylmethionine (SAM) and glutathione (GSH) levels, serum ALT and alkaline phosphatase (ALP) levels were significantly elevated suggesting that MCD diet-induced liver injury is different from that caused by ethanol. A significant synergistic interaction between MCD and ethanol results in exacerbated liver injury possibly due to increased hepatic steatosis and intense oxidative stress.

2. Materials and methods

2.1. *Animal treatment*

Age-matched male mice with the C57BL/6 background (10–12 weeks old) were used in all the experiments. The mice were housed individually in steel microisolator cages at 22 °C with a 12-h/12-h, light/dark cycle. After 2 days of feeding mice control liquid diet, mice were randomized into four dietary groups ($n = 6$). Each group of mice received one of four different liquid diets for 6 weeks: (1) paired-fed methionine-choline sufficient diet (control); (2) methionine-choline sufficient with ethanol (ethanol); (3) paired-fed methionine-choline deficient without ethanol (MCD); and (4) MCD plus ethanol (MCD + ethanol, or combination). Ethanol comprised 27.5% of total calories in mice receiving ethanol. Liquid diets were based upon the Lieber-DeCarli ethanol formulation and were purchased from DYETS Inc. (Bethlehem, PA). Protein content was 18.9% calories, fat was 16.5% of calories, and 64.5% of calories was carbohydrate. The MCD diet was similar to the control diet except complete removal of methionine and choline. For the control diet, methionine was provided at 1.18 g/L and choline at 0.66 g/L. When ethanol was included in the diet, 27.5% of calories from cornstarch were replaced with ethanol. The ethanol- and combination diet groups were allowed free access to ethanol-containing diets with increasing concentrations of ethanol over a 7-day period as previously described [20]. The ethanol concentration was kept thereafter at 5% for an additional 5 weeks. Animals received humane care and all procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Kansas University Medical Center Institutional Animal Care and Use Committee. After 6 weeks of feeding, mice were sacrificed. Blood samples were centrifuged

at 3000 rpm for 15 min to collect serum. Livers were rapidly excised and weighed. Livers were snap-frozen in liquid nitrogen and kept at −80 °C for RNA extraction, lipid peroxidation assay, lipid extraction or Oil Red O staining. Part of the livers was immediately homogenized for isolation of microsomes and cytosol or fixed in 10% formalin for hematoxylin and eosin (H & E) staining.

2.2. Serum ALT and ALP activities

Serum was stored at −80 °C and used for assay of ALT and ALP activities. Serum ALT activity was determined using Liquid ALT Reagent kit (Pointe Scientific Inc., Brussels, Belgium). Serum ALP activity was determined using ALP (Liquid) Reagent Set (Pointe Scientific Inc., Canton, MI).

2.3. Hepatic triglyceride, nonesterified fatty acid (NEFA) levels, and thiobarbituric acid-reactants contents

Hepatic lipids were extracted from 100 mg of liver homogenate using methanol and chloroform as previously described [21]. Hepatic triglyceride was quantified using a Triglyceride test kit (Wako pure Chemical Industries, Richmond, VA). Hepatic NEFA level was determined using the NEFA C test kit (Wako pure Chemical Industries, Richmond, VA). The extent of lipid peroxidation in the liver homogenate was quantitatively determined by measuring the concentration of the thiobarbituric acid (TBA)-reactive product, malondialdehyde (MDA) [22]. 1,1,3,3,-Tetramethoxypropane was used as an authentic standard for calibrating the MDA concentration against TBA reactivity.

2.4. H & E and Oil Red O staining of liver sections

Following fixation of the livers with 10% formalin/phosphate-buffered saline, livers were sliced and stained with H & E for histological examination. Frozen liver sections (10 μm) were stained with Oil Red O and counterstained with H & E for lipid content determination.

2.5. Preparation of cytosol and microsomes and measurement of GST and Cyp2e1 activity

Cytosolic and microsomal fractions were separated from fresh liver tissue as described previously [23]. Total GST activity in the cytosol was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as described previously [24]. Cyp2e1 activity in the liver microsomes was estimated colorimetrically by measuring the hydroxylation of *p*-nitrophenol to 4-nitrocathecol [25].

2.6. Quantification of GSH, S-SAM and S-adenosylhomocysteine (SAH)

Liver homogenate was prepared as described previously [26] and mixed with equal volume of 10% trichloroacetic acid solution and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was collected and stored at −80 °C. Total GSH was assayed by the recycling method [27]. The levels of SAM and SAH were measured as described previously [26].

2.7. Western blot analysis

Liver homogenate (25–50 μg/lane) were separated by 10–15% SDS-PAGE gels, electroblotted onto polyvinylidene difluoride membrane and immunoblotted with phosphospecific anti-signal transducer and activator of transcription factor 3 (STAT3) (Tyr 705) antibody (Cell Signaling Technology, Boston, MA) liver fatty acid binding protein (L-FABP), Bcl-2, TNFα, and IL-6 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with the appropriate peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies diluted in Tris-buffered saline with 0.1% Tween 20 (TBST) plus 1% non-fat dry milk for 1 h at room temperature. Following probing, blots were stripped and reprobed with anti-STAT3 (Cell Signaling Technology, Boston, MA), or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Proteins were viewed using enhanced chemiluminescence. Protein contents were determined by the Bradford method [28].

2.8. Determination of mRNA levels using real-time RT-PCR

Total RNA was isolated from frozen liver tissues using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbard, CA). RNA concentration and quality was determined spectrophotometrically at 260 nm and by the A260/A280 ratio, respectively. Total RNA (1 μg) was reversed transcribed into cDNA in a total reaction volume of 50 μl with the use of 2.5 U of Molony murine leukemia virus reverse transcriptase (MMLV-RT; Invitrogen, Carlsbard, CA) and 6.25 ng of oligo(dT)15 (Promega, Madison, WI) as a primer. The samples were placed in a Thermo Cycler and heated to 42 °C for 15 min and then to 95 °C for 15 min. The cDNAs were then diluted 10 times with water and subjected to RT-PCR to quantify Cyp4a14, glutathione peroxidase (GPX), methionine adenosyltransferase (MAT) 1A, MAT2A, L-FABP, acyl-CoA oxidase (AOX), β-actin, and GAPDH mRNA. RT-PCR was performed on an Applied Biosystems Prism 7900 Real-time PCR Instrument (Applied Biosystems, Foster City, CA) with the TaqMan fluorogenic probes (Applied Biosystems, Foster City, CA). Primers and probes (Table 1) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA). The primers and probes were designed to cross introns to ensure that only cDNA, but not genomic DNA, was amplified. The fluorogenic MGB probe was labeled with the reporter dye FAM (6-carborxyfluorescein). TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used to prepare the PCR mix. The amplification reactions were carried out with initial hold steps (50 °C for 2 min, followed by 95 °C for 10 min) and 40 cycles of a 2-step PCR (92 °C for 15 s, 60 °C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative CT method was used to determine fold differences between samples. The amount of mRNA was calculated using the comparative CT method which determines the amount of target normalized to an endogenous reference. Each gene was normalized to either GAPDH or β-actin after a validation experiment to verify that efficiency of the target gene amplification is equal to that of the endogenous reference.

Table 1 – Sequences of primers and probes used for real-time quantitative PCR

Name		Sequence	Accession #
L-FABP	Sense	TGCATGAAGGGAAGAAAATCAAA	NM017399
	Antisense	CCCCCAGGGTGAACTCATT	
	Probe	TCACCATCACCTATGGACCCAAAGTGG	
AOX	Sense	TTTGTTGTCCCTATCCGTGAGA	NM015729
	Antisense	GCCGATATCCCCAACAGTGA	
	Probe	TGGGACCCACAAGCCTCTGCCA	
β-Actin	Sense	CTTCTTTGCAGCTCCTTCGTTG	NM007393
	Antisense	CGACCAGCGCAGCGATATC	
	Probe	CCACACCCGCCACCAGTTCGCC	
MAT1A	Sense	TGATGAGACCGAGGAATGCA	NM133653
	Antisense	GATCTGCTATCCGGGTGTTGAG	
	Probe	CCCTTACCATCGTGCTCGCTCACAA	
MAT2A	Sense	CACAAGCTAAATGCCAAATTGG	NM145569
	Antisense	TTGAGTTTTAGAATCTGGGCGTAA	
	Probe	TGAACTACGCCGCAATGGTACATTGC	
Cyp4a14	Sense	CAAGACCCTCCAGCATTTCC	NM007822
	Antisense	GAGCTCCTTGTCCTTCAGATGGT	
	Probe	TGCATGCCTTCCCACTGGCTTTG	
GAPDH	Sense	TGTGTCCGTCGTGGATCTGA	NM001001303
	Antisense	CCTGCTTCACCACCTTCTTGA	
	Probe	CCGCCTGGAGAAACCTGCCA	
GPX	Sense	AGTGCGAAGTGAATGGTGAGAA	NM008160
	Antisense	GGTGTTGGCAAGGCATTCC	
	Probe	TCACCCGCTCTTTACCTTCCTG	

2.9. *Statistical analysis*

Data are presented as means ± S.E.M. Statistical analysis was performed using One-way ANOVA followed by Post Hoc tests. A P value of <0.05 was considered statistically significant.

3. Results

Six weeks after mice were fed ethanol, MCD, or combination diets, serum ALT activity was measured as an index of hepatocyte injury. While mice fed ethanol had no liver injury, significantly elevated serum ALT levels were observed in mice fed MCD diet (1.6-fold) and combination diet (10-fold) compared with mice fed the control diet (Fig. 1a). Serum ALP levels were significantly increased in mice fed MCD diet (1.9-fold) compared with mice fed control diet (Fig. 1b). Ethanol ingestion did not increase serum ALP levels. However, the combination diet significantly increased ALP levels by 1.6-fold compared with mice fed control diet. Both serum ALT and ALP levels were significantly increased in mice fed MCD or combination diets compared with mice fed ethanol alone (Fig. 1a and b).

Ethanol intake for 6 weeks had no apparent effect on body and liver weight compared with mice fed control diet (Table 2). However, ingestion of the MCD or combination diet resulted in

Table 2 – Effects of diets on mouse body and liver weight

Parameter	Unit	Control	Ethanol	MCD[a]	MCD + ethanol
Body weight	g	34.3 ± 3.4	31.3 ± 1.6	23.0 ± 2.5$^{***,\#\#}$	20.0 ± 1.1$^{***,\#\#\#,\S}$
Change in body weight	g	+1.08	+0.80	−9.50	−12.60
Absolute liver weight	g	1.27 ± 0.2	1.21 ± 0.1	0.79 ± 0.1$^{***,\#\#}$	0.93 ±± 0.2$^{**,\#}$
Liver/body weight	%	3.72 ± 0.5	3.86 ± 0.3	3.42 ± 0.2	4.62 ± 0.6$^{*,\#,\S\S}$

Data are mean ± S.D. for 5–6 mice per group.
*** $P < 0.001$ vs. mice on control diet.
** $P < 0.01$ vs. mice on control diet.
* $P < 0.05$ vs. mice on control diet.
$^{\#\#\#}$ $P < 0.001$ vs. mice on ethanol diet.
$^{\#\#}$ $P < 0.01$ vs. mice on ethanol diet.
$^{\#}$ $P < 0.05$ vs. mice on ethanol diet.
§§ $P < 0.01$ vs. mice on MCD diet.
§ $P < 0.05$ vs. mice on MCD diet.
[a] MCD, mice on methionine-choline deficient diet without ethanol.

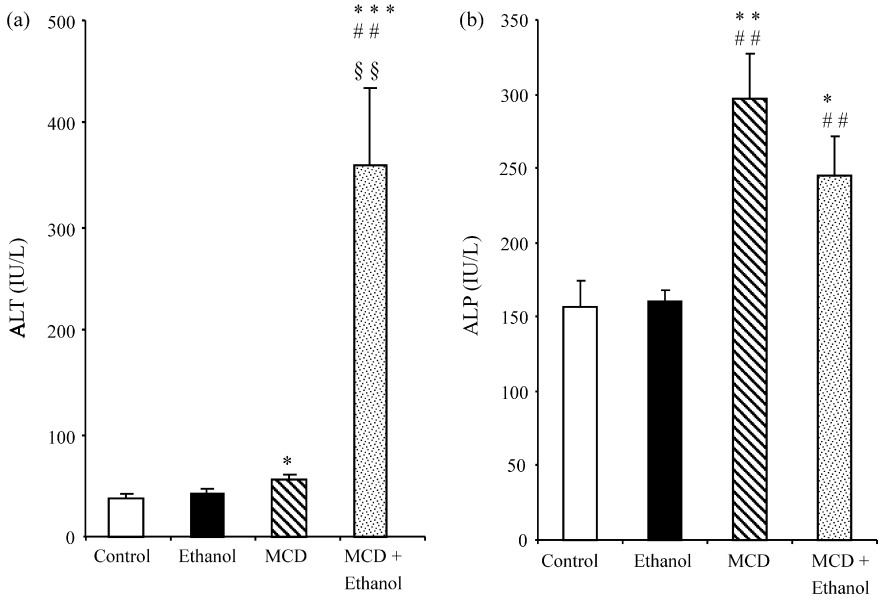

Fig. 1 – Serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities in mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed the control (pair-fed), ethanol, MCD (pair-fed) or combination diet for 6 weeks and ALT (a) and ALP (b) levels were determined. Data represents means ± S.E.M. (n = 5–6). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. mice the fed control diet. $^{\#\#}P < 0.01$ vs. mice fed the ethanol diet. $^{\S\S}P < 0.01$ vs. mice fed the MCD diet.

significant decreases in both body weight and absolute liver weight compared with mice fed control or ethanol diet (Table 2). When liver weight was expressed as percent of body weight, significant increase was only found in mice fed the combination diet (Table 2).

Administration of ethanol, MCD, or combination diet resulted in significant increases in liver triglycerides levels in comparison with control diet (Fig. 2a). However, hepatic NEFA levels were significantly increased only in mice fed combination diet (Fig. 2b). Histological analysis of the livers with H & E and Oil Red O staining revealed prominent lipid accumulation in the livers of mice fed the combination diet (Fig. 3d and h). Lipid accumulations were also found in the livers of ethanol-fed mice (Fig. 3b and f) or MCD (Fig. 3c and g)

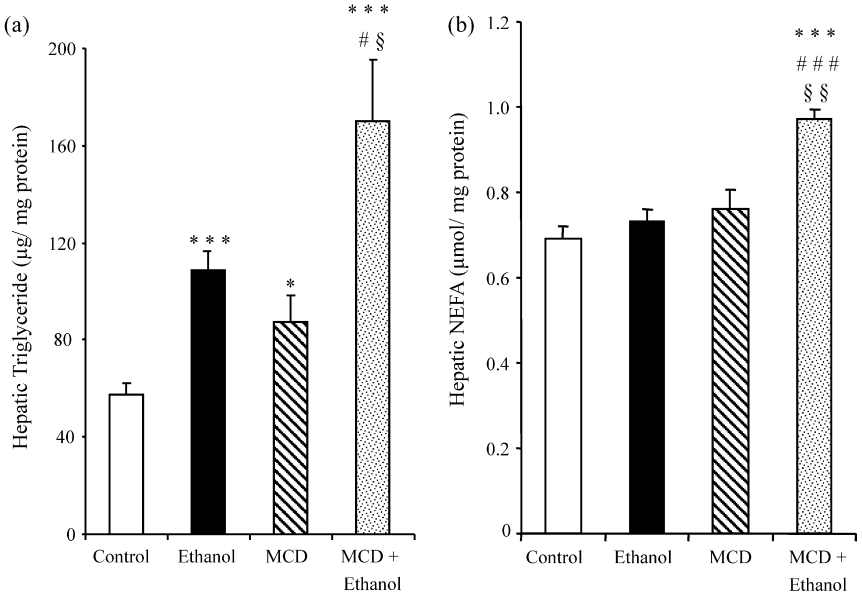

Fig. 2 – Hepatic triglyceride and nonesterified fatty acid (NEFA) levels in mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed the control (pair-fed), ethanol, MCD (pair-fed) or combination diet for 6 weeks. Hepatic triglyceride (a) and NEFA (b) levels were determined as described in Section 2. Data represents means ± S.E.M. (n = 6). $^{***}P < 0.001$, $^{*}P < 0.05$ vs. mice fed the control diet. $^{\#\#\#}P < 0.001$, $^{\#}P < 0.05$ vs. mice fed the ethanol diet. $^{\S\S}P < 0.01$, $^{\S}P < 0.05$ vs. mice fed the MCD diet.

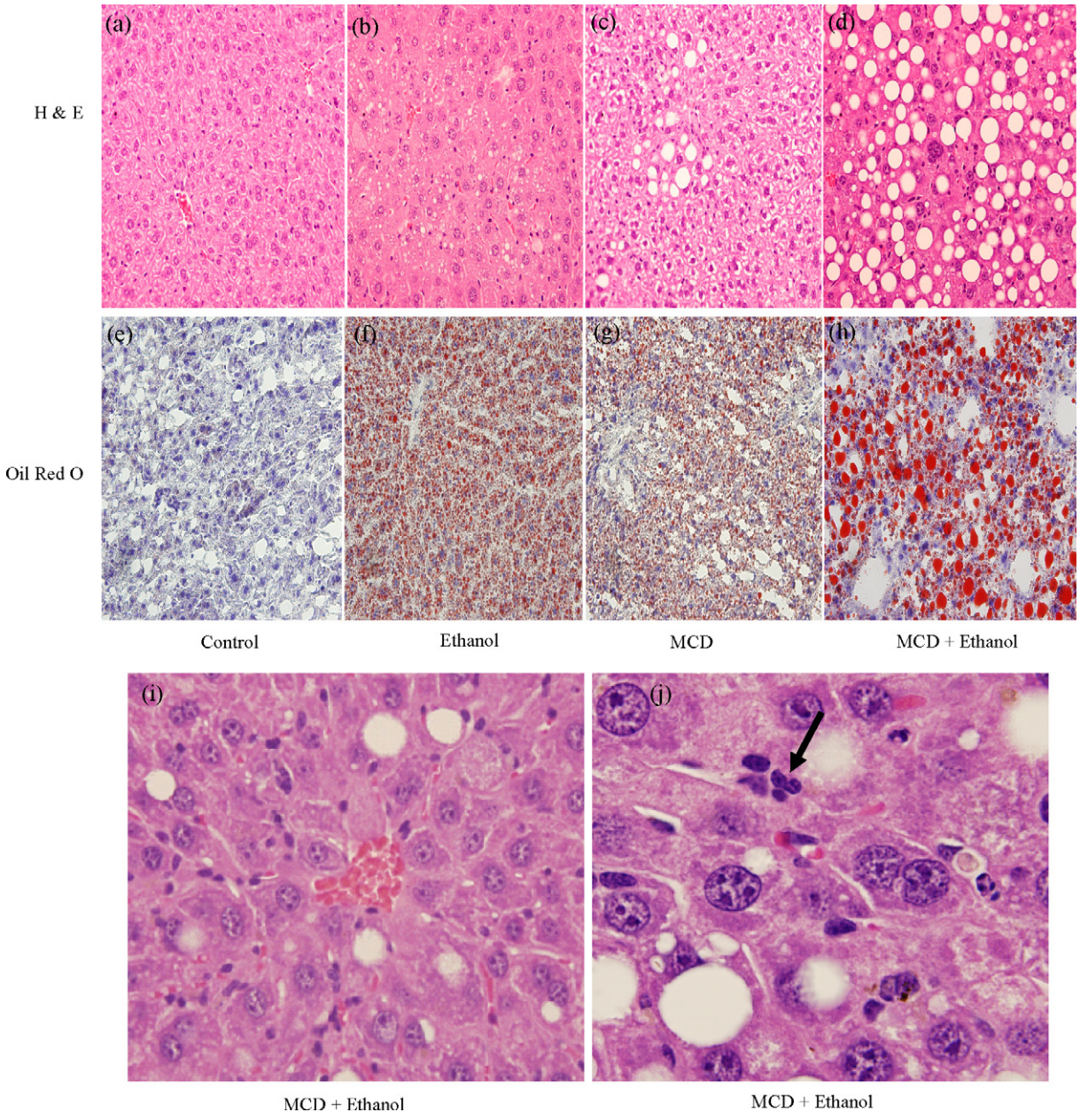

Fig. 3 – Histology of livers stained with H & E or Oil Red O of mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed the control (pair-fed, a and e), ethanol (b and f), MCD (pair-fed, c and g), or combination (d and h) diet for 6 weeks. H & E and Oil Red O stainings (original magnification ×400) from different treatment groups were performed as described in Section 2. In the livers of mice fed the combination diet, H & E staining revealed focal necrosis of hepatocytes (Fig. 3i) (original magnification ×400) and neutrophil infiltration (Fig. 3j) (original magnification ×1000).

diets, whereas lipid droplets were rare in the livers of the control group (Fig. 3a and e). In the livers of mice fed the combination diet, H & E staining revealed minor necrosis of hepatocytes and neutrophil infiltration (Fig. 3i and j).

Fig. 4 shows that mice fed ethanol exhibited significant increases in mRNA levels of hepatic L-FABP (6-fold) and AOX (3-fold) compared to control mice. However, MCD diet decreased the mRNA levels of L-FABP (0.4-fold) and AOX (0.2-fold) compared to control levels (Fig. 4a and b). Similarly the combination diet decreased L-FABP and AOX mRNA levels to 0.2-fold and 0.3-fold, respectively, compared to levels found in control mice (Fig. 4a and b). Consistent with increased mRNA levels by ethanol, Western analysis indicated that ethanol-fed mice had increased L-FABP protein levels (1.9-fold) (Fig. 4c and d). Although, L-FABP mRNA levels were decreased by MCD, the protein level was not affected (Fig. 4c and d). The combination diet however decreased L-FABP protein levels (Fig. 4c and d).

Mice fed the combination diet exhibited a significant increase in lipid peroxidation (LPO) using MDA as an index (Fig. 5a), however, mice fed ethanol, MCD or control diet did not exhibit an increase in LPO levels (Fig. 5a). Cyp2e1 has been

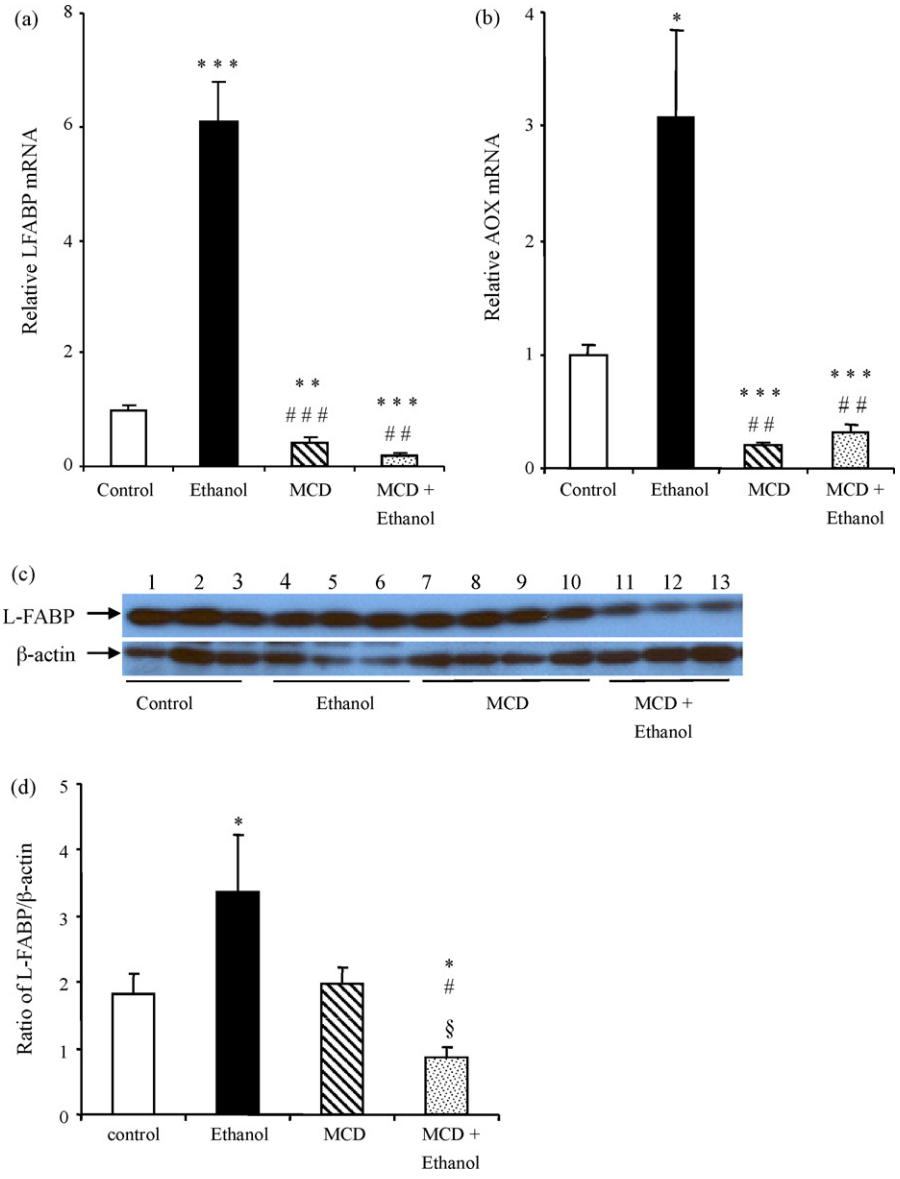

Fig. 4 – Liver fatty acid binding protein (L-FABP) and acyl-CoA oxidase (AOX) expression in mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed the control (pair-fed), ethanol, MCD (pair-fed) or combination diet for 6 weeks. L-FABP (a) and AOX (b) mRNA levels were determined as described in Section 2. Liver homogenate (25 μg/lane) was subjected to Western blotting and incubated with mouse anti-L-FABP antibody as described under Section 2 (c). Densitometry analysis was performed by the Gel-Pro Analyzer 3.1 Software and plotted after normalizing with β-actin (d). Data represents means ± S.E.M. (n = 3–6). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. mice fed control diet. $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ vs. mice fed ethanol diet. $^{§}P < 0.05$ vs. mice fed MCD diet.

shown to be involved in LPO and its levels are increased in rodents administered alcohol or MCD [29]. Cyp2e1 activity was significantly increased to a similar level in all treatment groups compared with the controls (Fig. 5b). It has been reported that hepatic Cyp 4a might be another source of LPO and might be induced by the MCD diet in addition to Cyp2e1 [30]. Since Cyp2e1 activities were increased in all treatment groups, we examined Cyp4a14 gene expression. Cyp4a14 mRNA levels were significantly increased 6.5-fold in ethanol-, 5.0-fold in MCD-, and 10-fold in combination-fed mice compared with mice fed the control diet (Fig. 5). While a greater induction of Cyp4a14 might contribute to increased generation of reactive oxygen species (ROS) and therefore LPO, the greater extent of LPO in the livers of mice treated with the combination diet compared with mice fed either ethanol or MCD diet does not mean LPO depends only on Cyp4a14. Oxidative stress is the result of an imbalance in the cellular ratio between pro-oxidant and antioxidant systems. Our results therefore indicate that the differences observed in MDA levels are the result of not only Cyp2e1 or Cyp4a14 or both and the ROS they generate, but more importantly, on the shift in the balance towards increased pro-oxidant generation compared with antioxidant systems.

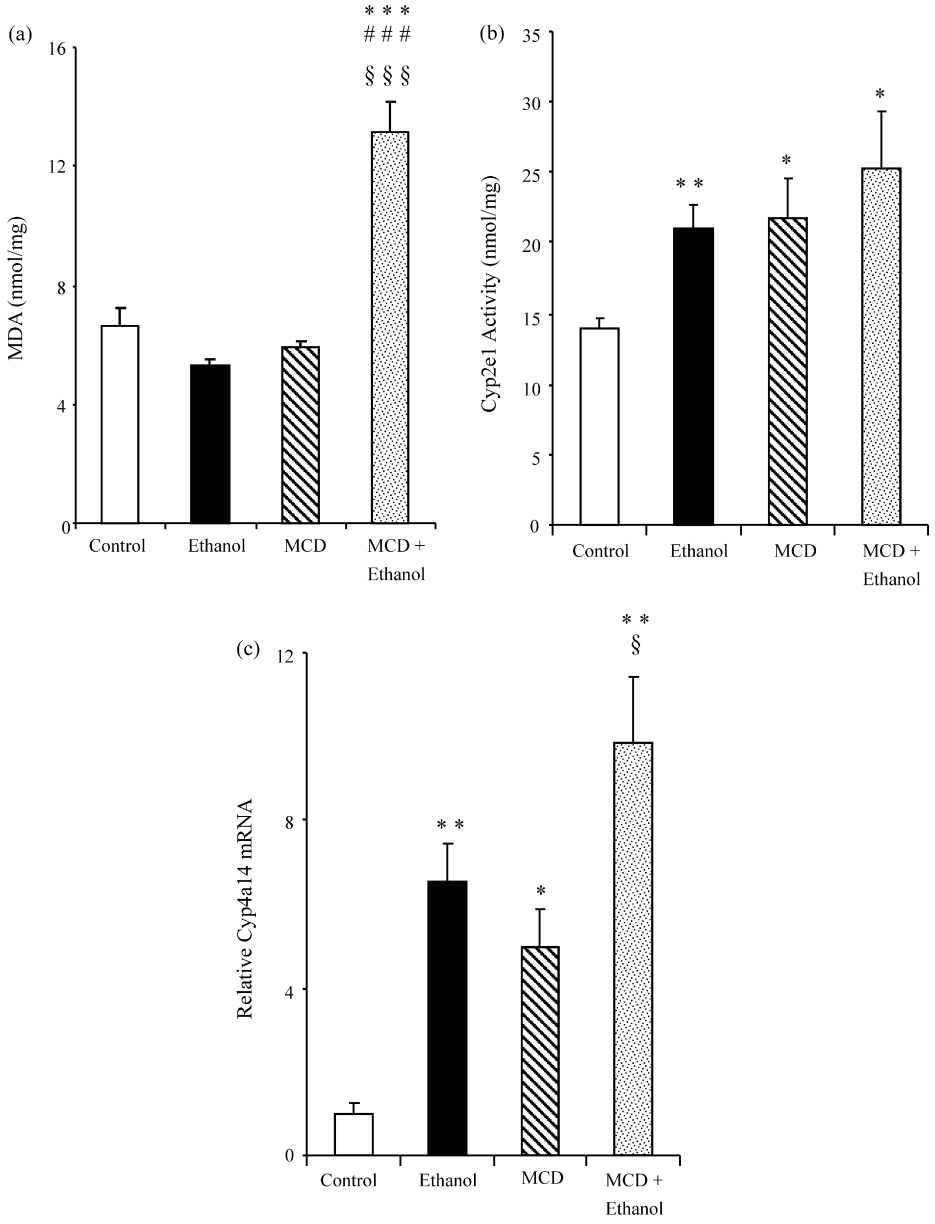

Fig. 5 – Lipid peroxide (LPO) levels, Cyp2e1 activity, and Cyp4a14 mRNA level in mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed the control (pair-fed), ethanol, MCD (pair-fed) or combination diet for 6 weeks. The production of LPO was estimated as malondialdehyde (MDA) (a), Cyp2e1 activities were assessed by the oxidation of *p*-nitrophenol to *p*-nitrocatechol (b), and Cyp4a14 mRNA levels (c) were determined by real-time PCR. Data represents means ± S.E.M. ($n = 4–6$). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. mice fed control diet. $^{\#\#\#}P < 0.001$, vs. mice fed ethanol diet. $^{§§§}P < 0.001$, $^{§}P < 0.05$ vs. mice fed MCD diet.

Cytosolic GST activities were also significantly increased in mice fed ethanol or combination diet, but not MCD diet compared with mice on the control diet (Fig. 6a). The GPX mRNA level was increased 2-fold in mice fed ethanol compared with mice fed the control diet (Fig. 6b). Ingestion of the MCD or combination diet did not increase GPX mRNA levels (Fig. 6b).

Ingestion of the ethanol or combination diets, but not MCD diet, resulted in a significant decrease in SAM levels (Fig. 7a). The SAH levels were significantly increased in all treatment groups compared with mice fed the control diet (Fig. 7b). Furthermore, the SAM-to-SAH ratios were significantly decreased in all treatment groups (Fig. 7c). Consistent with decreased SAM levels in mice ingesting ethanol, GSH levels were significantly decreased in ethanol-fed mice compared with mice fed control diet (Fig. 7d). The MCD or combination diets however did not significantly affect GSH levels in mice fed these diets compared with mice on control diet (Fig. 7d). We also examined the expression of MAT1A and MAT2A expression after ethanol, MCD, or combination treatment. As shown in Fig. 8, ethanol treatment did not affect MAT1 mRNA levels. Neither MCD nor combination treatment changed

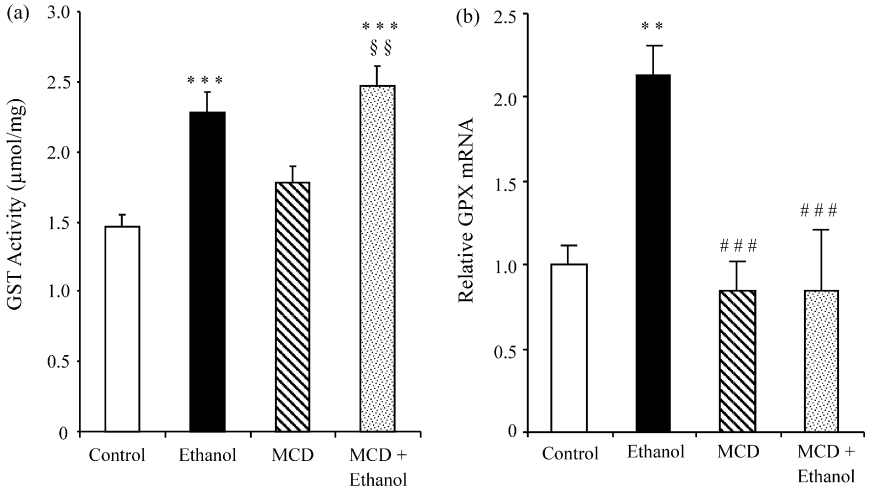

Fig. 6 – Hepatic glutathione S-transferase (GST) activities and glutathione peroxidase (GPX) mRNA levels in mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed control (pair-fed), ethanol, MCD (pair-fed) or combination diet for 6 weeks. GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (a). GPX mRNA levels (b) were determined by real-time PCR. Data represents means ± S.E.M. (n = 4–6). $^{***}P < 0.001$, $^{**}P < 0.01$ vs. mice fed control diet. $^{###}P < 0.001$ vs. mice fed the ethanol diet. $^{§§}P < 0.01$ vs. mice fed the MCD diet.

MAT1A mRNA levels. However, MAT2A mRNA level was significantly increased by all treatments (1.6- to 3.3-fold) (Fig. 8).

To determine whether the elevated ALT levels and massive steatosis observed after mice were fed the combination diet could be mediated by cytokines known to modulate inflammation, steatosis, and liver regeneration, TNFα and IL-6 protein levels were examined by Western blot. TNFα protein levels increased by 1.8-, 2.2-, and 1.5-fold in the livers of mice treated with ethanol, MCD, and combination diet, respectively, compared with mice fed the control diet (Fig. 9a and b). Unlike TNFα, IL-6 levels were increased only in ethanol-fed mice (2.1-fold). While IL-6 protein levels were not affected by the MCD diet, the combination diet decreased IL-6 levels to 0.62-fold compared with mice fed the control diet (Fig. 9a and c).

The signal transducer and activator of transcription factor 3 (STAT3) is a transcription factor implicated in the regulation of inflammatory signaling in the liver and plays a key role in hepatocyte proliferation and apoptosis prevention after liver injury [31,32]. Both Ethanol and MCD diets induced the level of phosphorylated STAT3 (Fig. 10a and b). In contrast, the combination treatment resulted in a reduction of STAT3 phosphorylation (Fig. 10a and b). While ethanol did not have any effect on the anti-apoptotic Bcl-2 protein, MCD increased Bcl-2 protein (2.2-fold) compared to mice fed control diet. The combination diet decreased Bcl-2 protein to 0.3-fold compared with control.

4. Discussion

In this study, we used feeding ethanol and MCD separately or together to compare the pathogenesis of ASH and NASH. Our results indicate that feeding ethanol or MCD separately induced steatosis and that the combination diet was associated with severe steatosis with liver injury. While ethanol ingestion alone did not produce liver injury, it did alter antioxidant defense mechanisms as well as induce the PPARα-regulated genes, L-FABP and AOX. Feeding the MCD diet, but not ethanol, increased serum ALP levels, suggesting that the MCD diet causes bile duct injury. A correlation between the lack of PPARα-target gene induction, decreased hepatic IL-6 expression, and severity of liver injury and elevated hepatic NEFA levels was observed when the mice were fed the combination diet. Furthermore, ingestion of ethanol, MCD, or combination diet increased the non-liver specific MAT2A mRNA expressions without a reduction in MAT1A mRNA levels.

Methionine and choline, important intermediates in various metabolic pathways, are precursors of phosphatidylcholine (PC) [33]. PC is an essential substrate for very low density lipoproteins (VLDL) synthesis [33]. When these nutrients are deficient, VLDL production or secretion, or both, are compromised [33,34]. Consequently, triglycerides accumulate in hepatocytes due to reduction in fatty acid export. Similarly, ethanol ingestion decreases PC synthesis by altering methionine metabolism leading to decreased SAM synthesis [35]. SAM is the principal methyl donor in methylation reactions. Methyl group transfer from SAM to phosphatidylethanolamine is necessary to generate PC. Furthermore, it has been shown that SAM is essential in the transport of fat from the liver, thereby preventing steatosis and consequently liver damage [36]. As expected, feeding ethanol or MCD led to increased hepatic triglyceride levels. The combination diet produced severe steatosis suggesting that exacerbation of triglyceride and fatty acid accumulation in the hepatocytes was promoted by PC deficiency, possibly due to the additive effect of MCD and ethanol. Indeed, hepatic free fatty acid levels were significantly increased in the livers of mice fed the combination diet, but not in mice fed either ethanol or the MCD diet alone. Intracellular accumulation of free fatty acids

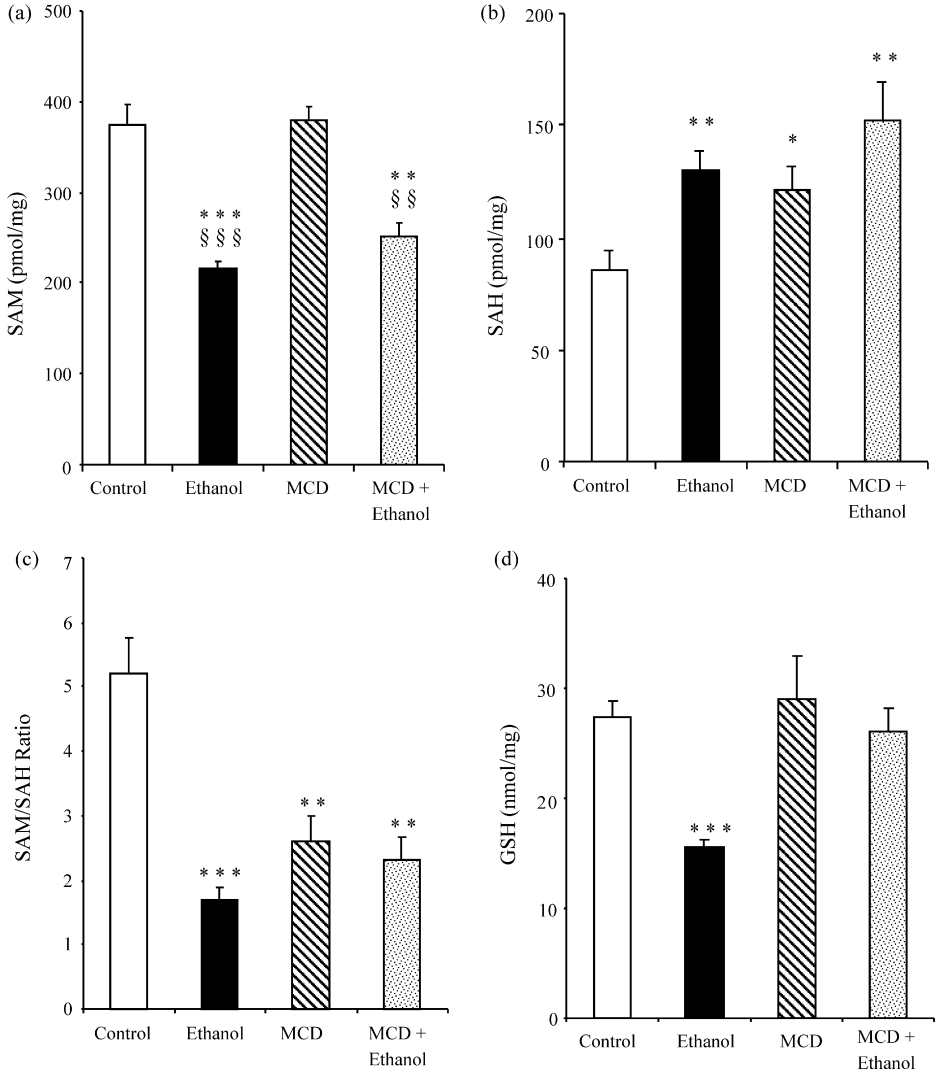

Fig. 7 – S-Adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), SAM/SAH ratio, and GSH levels of mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed the control (pair-fed), ethanol, MCD (pair-fed) or MCD + ethanol (combination) diet for 6 weeks. SAM levels (a), SAH levels (b), SAM/SAH ratio (c), and GSH levels (d) were determined as described in Section 2. Data represents means ± S.E.M. (*n* = 5–6). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. mice fed the control diet. $^{§§§}P < 0.001$, $^{§§}P < 0.01$ vs. mice fed the MCD diet.

may damage cell membranes and serve as a fuel for propagating lipid peroxidation [37]. The increased hepatic free fatty acid levels indicates impairment in free fatty acid catabolism, which is regulated by PPARα.

PPARα, a ligand-activated transcription factor that binds fatty acids, activates the transcription of genes that regulate lipid metabolism [38]. Activation of PPARα target genes, including L-FABP and AOX, by the PPARα agonist Wy14,643 ameliorated steatohepatitis induced by either ethanol or MCD [11,39]. Furthermore, PPARα-null mice fed either ethanol or MCD suffer more severe steatohepatitis [11,40]. The importance of PPARα-regulated genes in prevention of lipid abnormalities is further supported by the fact that mice deficient in the AOX gene develop severe steatosis and steatohepatitis [41]. When fatty acid levels increase, PPARα increases the catabolism of fatty acids by upregulating the expression of several genes involved in peroxisomal and mitochondrial β-oxidation and fatty acid transport [38]. In this study, increases in the mRNA and protein levels of L-FABP as well as AOX mRNA levels were observed in the ethanol fed mice, but significant decreases of the expression of these two genes were observed when mice were fed the combination diet. L-FABP regulates uptake of fatty acids, while AOX is the first and the rate-limiting enzyme involved in peroxisomal β-oxidation pathway [39,42]. Our results suggest that activation of these PPARα-target genes by ethanol produced hepatic fatty acid disposal, eventually preventing lipid overload, thereby depleting the liver of substrate for LPO and prevention of alcoholic steatohepatitis. Conversely, the lack of induction of these PPARα-regulated genes may be responsible for the liver injury observed in mice fed the combination diet.

Accumulation of fat in the liver provides the ''first hit'' in hepatic steatosis making the liver more susceptible to other insults which subsequently lead to liver injury [4]. Oxidative

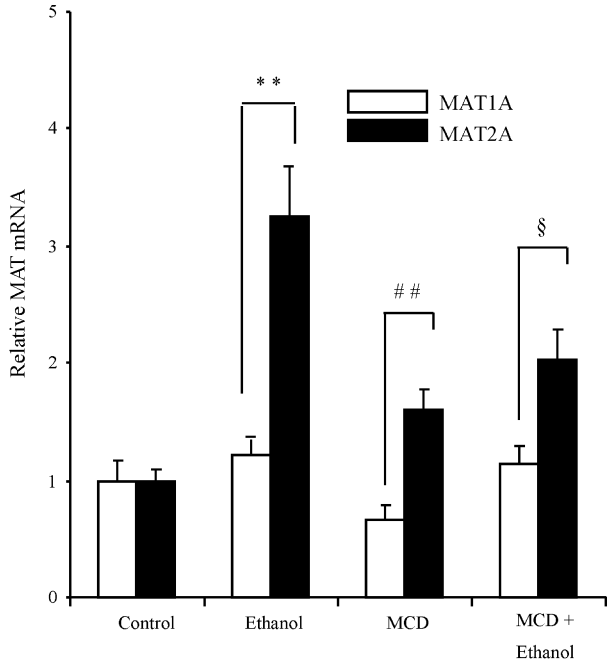

Fig. 8 – Methionine adenosyltransferase (MAT) 1A, and MAT2A mRNA levels of mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Mice were fed control (pair-fed), ethanol, MCD (pair-fed) or MCD + ethanol (combination) diet for 6 weeks. MAT1A and MAT2A mRNA levels were determined as described in Section 2. Data represents means ± S.E.M. (n = 4–6). $^{**}P < 0.01$ vs. mice fed ethanol diet. $^{\#\#}P < 0.01$ vs. mice fed the MCD diet. $^{\S}P < 0.05$ vs. mice fed the combination diet.

stress and LPO are generally considered as the most important pathogenic mechanisms for "the second hit" for converting hepatic steatosis to steatohepatitis [43]. In this study, we observed increased LPO only in the mice fed the combination diet. Besides increased Cyp2e1 enzyme activity, the combination diet induced Cyp4a14 to a much higher extent compared with ethanol or MCD alone diets suggesting the importance of Cyp4a14 in stimulation of LPO. During ethanol metabolism, the generation of NADH in the cytosol by ADH results in an increased NADH concentration in mitochondria [10]. Ethanol ingestion, therefore, inhibits mitochondrial β-oxidation, which is the main pathway for oxidation of fatty acids. Similarly, MCD diet-induced triglyceride accumulation inhibits mitochondrial β-oxidation [44]. An increase in Cyp4a14-ω-hydroxylation provides an alternative pathway for the disposition of fatty acids that accumulate due to impairment of mitochondrial β-oxidation [45]. Fatty acid metabolism by microsomal enzymes such as Cyp2e1 and Cyp4a14 generates ROS [45]. Thus the upregulation of the microsomal ω-oxidation process by the combination diet likely generated H_2O_2, ROS, and products of LPO. Taken together, these findings suggest an association between the severity of liver injury and oxidative stress.

Both the ethanol and MCD diets could mediate some of their effects by decreasing SAM synthesis. SAM biosynthesis is catalyzed by MAT. SAM also serves as a source of cysteine via the transsulfuration pathway for GSH synthesis, a major cellular antioxidant [46]. In mammals, two different genes, MAT1A and MAT2A encode the liver-specific MAT1A and the non-liver-specific MAT2A, respectively [47,48]. Reports indicates that under pathological conditions, such as liver cancer [49] or thioacetamide toxicity [50], there is a switch in the expression of MAT genes in the liver from MAT1A to MAT2A which correlates with a decrease in SAM levels [49]. MAT2A has been reported to have a higher affinity than MAT1A for methionine, a key substrate for SAM synthesis [51]. However, MAT2A activity is strongly inhibited by SAM [51]. Hence, SAM levels cannot increase when both isozymes are fully expressed. In the current study, while MAT1A mRNA levels remain unchanged, the mRNA levels of the non-liver-specific MAT2A were induced in the livers of mice fed ethanol, MCD, or combination diet. We speculated that the changes we observed in the MAT isozyme expression pattern after ethanol, MCD, and combination diets have an impact on SAM and SAH levels as well as the SAM/SAH ratios.

Our data revealed that MCD feeding did not decrease SAM levels, however, ethanol feeding or the combination diets resulted in decreased SAM levels. Consistent with the reduction of SAM levels in the ethanol-fed mice, the level of hepatic GSH, derived from SAM by the transsulfuration pathway, was also decreased in ethanol treated mice. As SAM is a precursor of GSH, it was expected that GSH levels will also decrease in the livers of mice fed the combination diet since SAM was decreased. Previous studies have suggested that ethanol exposure results in selective depletion of the mitochondrial pool of GSH [52]. It is therefore possible that the discrepancy we observed may result from measuring GSH levels in whole homogenate instead of mitochondria. Furthermore, both decreased and no decreased levels of GSH have been reported after ethanol exposure [52,53]. No plausible explanation currently exists as to why GSH levels were not decreased in the combination treatment. The lack of effect of the combination diet on hepatic GSH levels may be an adaptive response to free radical production that protects against tissue damage. The observed ethanol-induced reduction of SAM and GSH indicates the presence of a mechanistic link between ethanol and perturbed methionine metabolism as previously reported by others [54].

Ethanol also increased both cytosolic GST activity and GPX mRNA levels suggesting the occurrence of oxidative stress. In the context of the ethanol-induced changes cited above, it is proposed that the pathogenesis of steatohepatitis induced by ethanol involves severe oxidative stress possibly due to ROS generated during ethanol metabolism by both alcohol dehydrogenase and Cyp2e1 [29]. The fact that the MCD diet did not cause a reduction in SAM levels was unexpected, however, it may suggest a decrease in SAM utilization as reported after 9 weeks of exposure to CCl_4 [55]. Furthermore, SAM levels did not decrease when micropigs were fed a folate deficient diet for 14 weeks [56]. We observed that ethanol, MCD, or the combination diet all increased SAH levels and decreased the SAM/SAH ratio. Taken together, while chronic alcohol ingestion causes SAM depletion, SAH elevation, and decreased SAM/SAH ratio, our results revealed that feeding MCD to mice causes fatty liver similar to alcohol ingestion without SAM depletion. This suggests a difference in the pathogenesis of

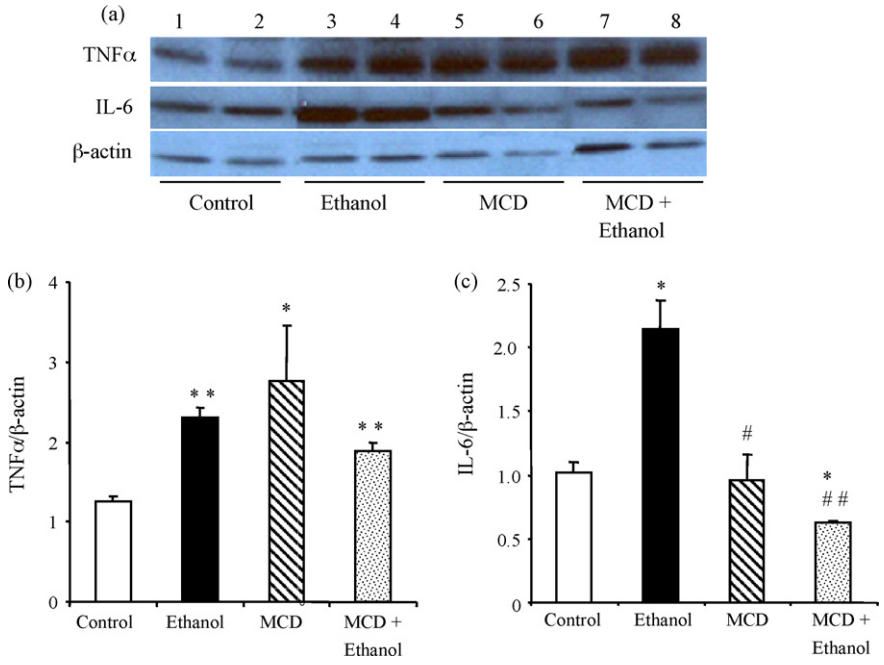

Fig. 9 – Immunoblot analysis of hepatic TNFα and IL-6 of mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Liver homogenate (50 μg/lane) from mice fed the control (pair-fed), ethanol, MCD (pair-fed) or MCD + ethanol (combination) diet were separated on 15% SDS-polyacrylamide, transferred to polyvinylidene difluoride membranes, and incubated with TNFα and IL-6 antibodies as indicated in representative blots (a). Quantitative analysis of TNFα (b) and IL-6 (c) levels plotted after normalizing with β-actin. Densitometry analysis was performed using the Gel-Pro Analyzer 3.1 Software. Data represents means ± S.E.M. (n = 3–4). $^{**}P < 0.01$, $^{*}P < 0.05$ vs. wild type mice fed control diet. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. mice fed ethanol diet.

ASH and NASH. The lack of effect of the MCD diet on the SAM and GSH pathway provides a possible difference between ASH and NASH in terms of the mediators involved in the propagation of steatohepatitis (Table 3).

Among the consequences of the decreased SAM levels in the livers of mice fed ethanol or the combination diet are an enhanced production of pro-inflammatory cytokines [20,57]. SAM concentrations have also been shown to critically modulate monocyte/Kupffer cell TNFα production [58]. In a previous report, it was shown that an alcohol-induced increase in TNFα production and a lack of induction of IL-6 in ethanol-fed female rats contribute to enhanced sensitivity of female liver to ethanol hepatotoxicity [59]. While elevation of IL-6 has been implicated in the severity and progression of both alcoholic and non-alcoholic steatohepatitis, increasing evidence indicate that IL-6 can prevent liver injury and apoptosis by promoting liver regeneration [31,60,61]. IL-6 is also known to decrease fat accumulation which serves as a fuel for LPO [62]. We show that while hepatic TNFα levels were increased in all treatment groups, IL-6 levels was induced only in the livers of mice fed ethanol. The increased hepatic IL-6 levels was associated with lack of ALT induction in the ethanol-fed mice suggesting that up-regulation of hepatic IL-6 levels was involved in protecting against liver injury. In contrast, lack of IL-6 induction was associated with liver injury in mice fed the MCD diet and decreased IL-6 levels in mice fed the combination diet correlated with massive steatosis and severe liver injury. Our data suggest that elevated hepatic IL-6 by ethanol serves as a protective cytokine against liver injury induced by ethanol. It is therefore suggested that decreased L-FABP protein, SAM, and IL-6 levels coupled with increased free fatty acids in addition to the high Cyp4a14 mRNA levels may in part lead to the formation of MDA products and liver injury we found in the livers of mice fed the combination diet.

Administration of the MCD or combination diets also caused significant increases in both serum bile acids (results not shown) and ALP levels. Our findings indicate that besides steatosis, the MCD diet may cause bile duct damage and cholestasis in these mice. Elevated levels of ALP are seen in subjects with NASH, however there are no reports indicating that the MCD diet alone can induce cholestasis [63]. Our results indicating that feeding the MCD diet can increase biochemical markers for cholestasis such as serum bile acids and ALP levels is important and needs further investigation.

Emerging data suggest that hepatocyte apoptosis may be a key component of the ''second hit'' involved in the progression of a simple steatosis to NASH [64]. STAT3 phosphorylation is a known anti-apoptotic pathway that protects the liver from ethanol-induced liver injury [32]. We observed that both ethanol and MCD diets induced the level of phosphorylated STAT3 while the combination diet caused a reduction. Bcl-2 protein which protects against toxicant-induced apoptosis was increased in the livers of mice fed the MCD diet, but decreased in mice fed the combination diet. This suggested that increased Bcl-2 levels led to hepatocyte survival in the mice fed the MCD diet resulting in mild liver injury compared

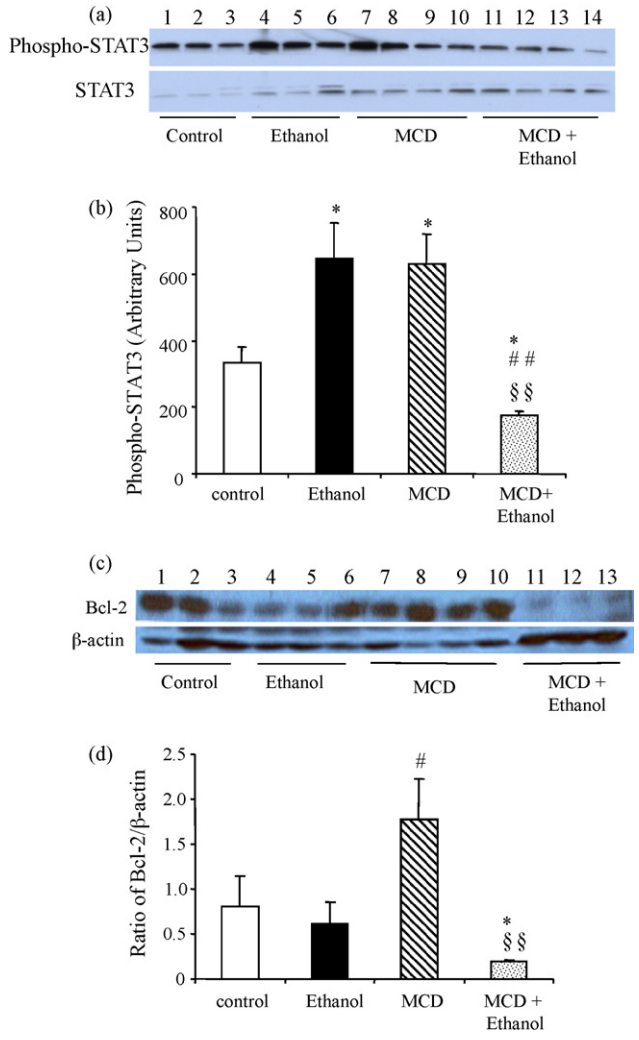

Fig. 10 – Immunoblot analysis of STAT3 phosphorylation and Bcl-2 in mice fed the control, ethanol, MCD or MCD + ethanol (combination) diet. Liver homogenate (50 μg/lane) from mice fed the control (pair-fed), ethanol, MCD (pair-fed) or MCD + ethanol (combination) diet were separated on 10% SDS-polyacrylamide, transferred to polyvinylidene difluoride membranes, and incubated with phosphospecific anti-STAT3 (Tyr 705), STAT 3 (a) and Bcl-2 (c) antibody. Quantitative analysis of phospho-STAT3 (b) and Bcl-2 (d) levels plotted after normalizing with β-actin. Densitometry analysis was performed using the Gel-Pro Analyzer 3.1 Software. Data represents means ± S.E.M. (n = 3–4). $^{*}P < 0.05$ vs. wild type mice fed control diet. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. mice fed ethanol diet. $^{\S\S}P < 0.01$ vs. mice fed MCD diet.

Table 3 – The effect of ethanol, MCD, and MCD + ethanol diets on biochemical parameters of mice

Parameter	Treatment		
	Ethanol	MCD	MCD + ethanol
Liver injury			
ALT	—	↑	↑↑
ALP	—	↑	↑
Lipid genes and levels			
L-FABP mRNA	↑	↓	↓
L-FABP protein	↑	—	↓
AOX mRNA	↑	↓	↓
Triglycerides	↑	↑	↑↑
Nonesterified fatty acids	—	—	↑
Antioxidant factors and genes			
GSH	↓	—	—
SAM	↓	—	↓
SAH	↑	↑	↑
SAM/SAH ratio	↓	↓	↓
GST	↑	—	↑
GPX	↑	—	—
MAT1A mRNA	—	—	—
MAT2A mRNA	↑	↑	↑
Lipid peroxidation			
Cyp2e1	↑	↑	↑
Cyp4a14	↑	↑	↑↑
Lipid peroxides (MDA)	—	—	↑↑
Anti-apoptotic signal			
Phospho-STAT3	↑	↑	↓
Bcl-2	—	↑	↓↓
Cytokines			
TNFα	↑	↑	↑
IL-6	↑	—	↓

with mice fed the combination diet. Our results revealed that reduced IL-6 and STAT3 signaling coupled with decreased Bcl-2 expression correlated with increased liver injury in the livers of mice fed the combination diet. Thus, the combination diet might reduce the STAT3-mediated anti-apoptotic effect. This may alter many signaling pathways involved in tissue repair and liver regeneration. The decreased IL-6, phosphorylated-STAT3, and Bcl-2 protein levels in the livers of mice treated with the combination diet suggested that in addition to oxidative stress, apoptosis might be another mechanism of liver injury. However, activated caspase 3 was not detected by Western blot (data not shown) at the time when mice were sacrificed. Apoptotic cells were also not present by H & E staining suggesting that apoptosis might not be a major mechanism of liver injury in this model. Another possibility is that the process of apoptosis might have been completed resulting in liver injury after 6 weeks of treatment. Shorter treatment duration is possibly required to determine the involvement of apoptosis in liver injury induced by the combination diet.

In summary, the present study demonstrates that chronic ethanol exposure without liver injury promotes severe oxidative stress seen as decreased hepatic SAM and GSH levels due to increased GSH utilization through the GST-GPX pathways. Furthermore, the transition from steatosis to steatohepatitis in the ethanol, MCD or combination diets are dependent on activation of PPARα regulated genes important for fatty acid catabolism and hepatic IL-6 induction. Thus, it is concluded that since steatohepatitis is magnified by a concomitant ingestion of MCD diet with ethanol, the use of this combination diet may provide beneficial insights into the pathogenesis of both ASH and NASH. Our data also provides an explanation for the heightened risk of steatohepatitis for NASH patients who drink alcohol or vice versa. Administration of PPARα-agonist to improve fatty acid oxidation is

needed to clarify the mechanism of steatohepatitis during the administration of the combination diet.

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