Heme oxygenase-1 alleviates alcoholic liver steatosis: histopathological study

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Abstract: Excessive alcohol consumption is one of the most important causes of hepatic steatosis, which involves oxidative stress. In particular, increased oxidative stress has been strongly linked to stimulation of the expression of heme oxygenase-1 (HO-1). This study aimed to investigate whether HO-1 could alleviates alcoholic steatosis in rats. Male Wistar rats were randomly divided into 4 groups: 1) the control group, 2) the EtOH group, 3) the EtOH + ZnPP-IX group and 4) the EtOH + Hemin group. Liver histopathology was investigated in weeks 1 and 4 after the start of the treatment period. Alcohol treatment significantly increased the hepatic malondialdehyde (MDA) levels, an oxidative stress marker. In addition, it increased the triglyceride, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in both weeks. Gross examination demonstrated a yellowish and slightly enlarged liver in the alcohol-treated rats. Hematoxylin and eosin (H&E) and Oil Red O staining indicated hepatic steatosis, which was characterized by diffuse, extensive fatty accumulation and discrete lipid droplets of variable size in hepatocytes of the alcohol-treated rats. Administration of the HO-1 inducer hemin resulted in upregulation of hepatic HO-1 gene expression, reduced the MDA, triglyceride, ALT and AST levels and alleviated alcoholic hepatic steatosis, whereas administration of the HO-1 inhibitor zinc protoporphyrin IX (ZnPP-IX) resulted in downregulation of hepatic HO-1 gene expression and could not alleviate alcoholic hepatic steatosis either week. In conclusion, HO-1 could alleviates alcoholic hepatic steatosis in male Wistar rats and may be useful in development of a new therapeutic approach. (DOI: 10.1293/tox.2015-0035; J Toxicol Pathol 2016; 29: 7–15)

Key words: heme oxygenase-1, alcoholic steatosis, zinc protoporphyrin IX, hemin, oxidative stress

Introduction

The liver is one of the main targets of alcohol-induced injury, as it is the principal organ of alcohol metabolism. Excessive and chronic alcohol consumption leads to alcoholic liver disease including steatosis, hepatitis and cirrhosis, which is associated with high mortality worldwide. Steatosis is present in more than 90% of binge and chronic drinkers1. Although liver steatosis is a reversible injury, its progression can develop into more severe liver problems including steatohepatitis and cirrhosis2. Oxidative stress has been suggested to play a vital role in mechanisms of alcoholic steatosis3. Several antioxidant molecules have been used in a novel and potential strategies to improve steatosis induced by ethanol in rodents4, 5. Moreover, increased oxidative stress has been strongly linked to stimulation of the expression of heme oxygenase-1 (HO-1)5. Several studies found that HO-1 could protect against ethanol-induced liver damage6, 7. HO-1 is highly concentrated in tissues that are heavily involved in the catabolism of heme proteins. It is a stress responsive protein that acts as the rate-limiting enzyme in the catabolism of pro-oxidant heme to produce equimolar amounts of carbon monoxide (CO) and biliverdin/bilirubin and release free iron8. Overproduction of biliverdin and bilirubin serves as an antioxidative mechanism9. Free iron inhibits the de novo synthesis of heme, binds to iron regulatory protein and stimulates the biosynthesis of ferritin, which exerts an additional antioxidant effect10. However, there is no research that has focused on the effect of HO-1 on alcoholic steatosis. Therefore, this study aimed to investigate whether the potent antioxidant HO-1 could alleviates alcoholic steatosis in male Wistar rats.
Materials and Methods

Chemicals
Bradford assay kits, and 10% neutral buffered formalin solution were purchased from Sigma-Aldrich corporation (St. Louis, MO, USA). A thiobarbituric acid reactive substances (TBARS) assay kit was obtained from Cell Biolabs, Inc (San Diego, CA, USA). Oil Red O dye was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Experimental design
Twenty-four male Wistar rats (Rattus norvegicus) aged 12 weeks were obtained from the Division of Animal House, Faculty of Science, Prince of Songkla University, Thailand. All animal procedures were reviewed and approved by the Animal Ethics Committee, Walailak University (Protocol number: 003/2014), and were conducted according to the Guide for the Care and Use of Laboratory Animals, National Research Council. Rats were maintained in stainless steel cages under constant conditions of temperature (23 ± 2°C), relative humidity (50–60%) and lighting (12 h light/dark cycles). Animals were provided with a standard commercial rat diet and distilled water. They were acclimatized and closely monitored under laboratory conditions for 2 weeks before the commencement of the experiment.

The male Wistar rats were randomly divided into 4 groups (6 rats per group): 1) the control group, in which rats were subjected to gavage with normal saline; 2) the EtOH group, in which rats were subjected to gavage with ethanol (30% v/v in saline, 5 g/kg, every 24 h) for 4 weeks; 3) the EtOH + ZnPP-IX group, in which rats were subjected to gavage with ethanol and intraperitoneally (i.p.) injected with the HO-1 inhibitor zinc protoporphyrin IX (ZnPP-IX) (20 µmol/kg) every week for 4 weeks; and 4) the EtOH + Hemin group, in which rats were subjected to gavage with ethanol and i.p. injected with the HO-1 chemical inducer hemin (30 µmol/kg) every week for 4 weeks. The procedure for alcohol feeding was based on the protocol of Nan et al.11. In weeks 1 and 4 after the start of the treatment period, rats were anesthetized with thiopental sodium i.p. [50 mg/kg body weight (BW)]. Peripheral blood from the heart was collected in heparinized tubes. Then, rats were euthanized with an anesthetizing thiopental sodium overdose (100 mg/kg BW). After opening the abdominal cavity, the liver was collected and immediately washed in ice-cold isotonic saline. The liver was weighed and fixed in 10% neutral buffered formalin solution for 24 h and was then placed in small metal caskets, stirred by a magnetic stirrer, dehydrated using alcohol series from 70% to 100% alcohol and embedded using alcohol series from 70% to 100% alcohol and embedded in paraffin using an embedding machine. The paraffin block was sectioned using a rotary ultramicrotome, distributed onto glass slides and then dried overnight. Slides were observed under a light microscope after being stained with hematoxylin and eosin (H&E) dyes and mounted.

Oil Red O staining
Frozen section of the liver was stained according to the following steps: deionized water (3 min), 60% isopropanol alcohol (2 min), Oil Red O dye (30 min) and deionized water (1 min). Afterwards, slides were covered with glycerol gel. The % of tissue covered by lipid droplets was calculated12.

Determination of hepatic MDA levels
The liver tissue was homogenized to give a final concentration of 50 mg/mL in phosphate buffered saline (PBS) containing 1X butylated hydroxytoluene (BHT), homogenized on ice and centrifuged at 10,000 × g for 5 min to collect the supernatant. In accordance with the protocol of an OxiSelect™ TBARS Assay Kit (Cat No. STA-330, Cell Biolabs, San Diego, CA, USA), 100 µL of the samples and 100 µL of the MDA standard were added to separate microcentrifuge tubes, and then 100 µL of the SDS lysis solution was added and mixed thoroughly. The samples were then incubated for 5 min at room temperature, 250 µL of thiobarbituric acid (TBA) reagent was added, and then each tube was closed and incubated at 95°C for 60 min. The tubes were then removed and cooled to room temperature in an ice bath for 5 min. All sample tubes were then centrifuged at 3000 rpm for 15 min, the supernatant was removed from the samples, and then finally 200 µL of each of the samples and 200 µL of the MDA standard were transferred to a 96-well microplate compatible with a spectrophotometric plate reader. The absorbance was read at 532 nm.

Liver histology
Histopathology of the tissue was performed according to the guidelines of the Department of Pathobiology, Faculty of Science, Mahidol University. The liver tissue was preserved in 10% neutral buffered formalin solution for 24 h and was washed with 70% ethanol. Tissue was then placed in small metal caskets, stirred by a magnetic stirrer, dehydrated using alcohol series from 70% to 100% alcohol and embedded in paraffin using an embedding machine. The paraffin block was sectioned using a rotary ultramicrotome, distributed onto glass slides and then dried overnight. Slides were observed under a light microscope after being stained with hematoxylin and eosin (H&E) dyes and mounted.

Biochemical analysis
Blood samples were centrifuged at 3000 rpm for 5 min. Sera were collected, and the levels of triglyceride, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a Cobas Mira Plus CC Chemistry Analyzer (Switzerland).
The primers for the HO-1 were 5′-TTTGGAAATGTGTCACAGAGG-3′, forward, and 5′-CAGGCAGAGAATGCTGAGTTC-3′, reverse, and those for beta actin were 5′-TTCTTTGCAGCTCCTTCGGTGC-3′, forward, and 5′-TGGATGGCTACGTACATGGCTGGG-3′, reverse. The sample was equally loaded on 2% gel agarose, stained with ethidium bromide and visualized with a UV transilluminator. The amount of PCR product was quantified using the GeneTools and image analysis software (Syngene, Frederick, MD, USA).

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Differences between groups were determined by one-way analysis of variance (ANOVA). Post hoc testing was performed for group comparisons using the Least Significant Difference (LSD) test, and \( P<0.05 \) was considered significant.

Results

Hepatic HO-1 mRNA expression

The ethanol-treated rats demonstrated increased hepatic HO-1 expression compared to control group (not significant). Administration of the HO-1 inducer hemin significantly upregulated hepatic HO-1 gene expression \((P<0.05)\), whereas administration of the HO-1 inhibitor ZnPP-IX downregulated hepatic HO-1 gene expression compared with the control group \((P<0.05)\), as shown in Fig. 1.

Effect of HO-1 on body weight

Rat body weight was significantly reduced in the ethanol consumption group in all weeks (week 1, \( P=0.003 \); week 2, \( P=0.007 \); week 3, \( P=0.003 \); and week 4, \( P=0.009 \)) compared with the control group. Moreover, rats in the EtOH + ZnPP-IX group had significantly reduced BWs in all weeks (week 1, \( P=0.003 \); week 2, \( P=0.004 \); week 3, \( P=0.002 \); and week 4, \( P=0.006 \)) compared with rats in the control group, as illustrated in Fig. 2. Surprisingly, rats treated with the HO-1 inducer hemin (30 \( \mu \)mol/kg) following ethanol administration showed significantly increased BWs compared with the EtOH and EtOH + ZnPP-IX groups in weeks 1 \((P=0.038, P=0.030, \text{respectively})\), week 3 \((P=0.041, P=0.034, \text{respectively})\), and week 4 \((P=0.033, P=0.023, \text{respectively})\).

Effect of HO-1 on biochemical parameters

As shown in Fig. 3, rats in the ethanol consumption groups had significantly increased triglyceride levels in both weeks 1 and 4 \((P<0.001)\), ALT levels in both weeks 1 and 4 \((P<0.05, P<0.001, \text{respectively})\) and AST levels in both weeks 1 and 4 \((P<0.05)\) compared with the rats in the control group. Rats in the EtOH + ZnPP-IX group, which was administered the HO-1 inhibitor ZnPP-IX (20 \( \mu \)mol/kg) following ethanol treatment, also demonstrated significantly increased triglyceride \((P<0.001)\), ALT \((P<0.05)\), and AST \((P<0.05)\) levels in both weeks 1 and 4 compared with rats in the control group. Rats treated with HO-1 the inducer hemin had significantly decreased triglyceride levels in weeks 1 and 4 \((P<0.05, P<0.001, \text{respectively})\) and significantly decreased ALT levels in week 1 \((P<0.05)\) and week 4 \((P<0.05, P<0.05, \text{respectively})\) compared with the EtOH group and EtOH + ZnPP-IX group, significantly decreased ALT levels in week 1 \((P<0.05)\) compared with the EtOH group and EtOH + ZnPP-IX group, significantly decreased ALT levels in week 4 compared with the EtOH group and EtOH + ZnPP-IX groups \((P<0.05, P<0.05, \text{respectively})\) and significantly decreased AST levels compared with the EtOH and EtOH + ZnPP-IX groups in both weeks \((P<0.05)\).

Effect of HO-1 on hepatic MDA levels

The EtOH and EtOH + ZnPP-IX groups had significantly increased hepatic MDA levels in both weeks 1 and 4 compared with the control group \((P<0.001)\), as shown in
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Fig. 4. Rats treated with hemin had significantly reduced MDA levels compared with the EtOH group and EtOH + ZnPP-IX group in both weeks (P<0.05).

**Effect of HO-1 on liver pathology**

Macroscopic examination of the liver in the control group showed normal tissue in both weeks 1 and 4 (Fig. 5A and 5B). The rat administered ethanol (4 g/kg/day) showed a yellowish and slightly enlarged liver in both weeks (Fig. 5C and 5D), and the rats in the EtOH + ZnPP-IX group, which was administered the HO-1 inhibitor ZnPP-IX following ethanol treatment, showed similar results (Fig. 5E and 5F). Fortunately, the rats treated with hemin following ethanol administration showed normal livers in both weeks as shown in Fig. 4G and 4H.

H&E staining of the liver in the control group revealed a normal histological structure. The ethanol consumption led to diffuse and extensive fatty vacuolation in hepatocytes. Moreover, ethanol induced variation in the size of lipid droplets depending on time of ethanol exposure, with several small fat vacuoles in the cytoplasm of hepatocytes or steatosis being observed in week 1 (Fig. 6), while accumulation of several large fat vacuoles in the cytoplasm of hepatocytes or macrovesicular steatosis that moved the nucleus towards the periphery of cells was observed in week 4 of ethanol exposure (Fig. 7). Administration of the HO-1 inhibitor ZnPP-IX following ethanol treatment also resulted in fatty vacuolation in both weeks. Liver steatosis was reversed by administration of the HO-1 inducer hemin.

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**Fig. 3.** Effect of HO-1 on levels of triglyceride (A), ALT (B) and AST (C) of male Wistar rats in weeks 1 and 4. Values are present as the mean ± SEM (per group n = 6). a,b Compared with the control group in weeks 1 and 4, respectively. c,d Compared with the EtOH group in weeks 1 and 4, respectively. * P<0.05. ** P<0.001.

**Fig. 4.** Effect of HO-1 on hepatic MDA levels of male Wistar rats in weeks 1 and 4. Values are present as the mean ± SEM (per group n = 6). a** Compared with the control group in week 1. b,c,d Compared with the EtOH group in weeks 1 and 4, respectively. e,f,g,h Compared with the EtOH + ZnPP-IX group in weeks 1 and 4, respectively. * P<0.05. ** P<0.001.
Fig. 5. Effect of HO-1 on liver morphology of male Wistar rats of the control group in weeks 1 (A) and 4 (B), the EtOH group in weeks 1 (C) and 4 (D), the EtOH + ZnPP-IX group in weeks 1 (E) and 4 (F) and the EtOH + Hemin group in weeks 1 (G) and 4 (H). Scale = centimeters.
Effect of HO-1 on liver steatosis using Oil Red O staining

As shown in Fig. 8, the EtOH group and EtOH + ZnPP-IX group showed significantly increased lipid droplet accumulations in the cytoplasm of hepatocytes compared with the control group ($P<0.001$). The administration of hemin following ethanol treatment had significantly reduced lipid droplet accumulations in hepatocytes compared with EtOH group and EtOH + ZnPP-IX group ($P<0.05$ and $P<0.001$, respectively).

**Discussion**

Alcoholic liver disease (ALD) results from excessive alcohol intake, and its clinical pathology includes fatty liver or steatosis, hepatitis and cirrhosis, which might make it possible to develop hepatocellular carcinoma. Progression of ALD involves multiple factors (sex, obesity and genes). More than 90% of heavy drinkers develop steatosis, and about 30% of alcoholics develop severe forms. The liver sustains the greatest damage after alcohol consumption because it is a principal organ of ethanol oxidation. Hepatic ethanol oxidation to acetaldehyde proceeds by two major pathways: alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1). Increased CYP2E1 in response to high concentrations of ethanol leads to production of reactive oxygen species (ROS) such as superoxide radical ($\text{O}_2^{-}$), hydroxyl radical (OH) and hydrogen peroxide ($\text{H}_2\text{O}_2$), which ultimately causes oxidative stress. Oxidation products of lipid, protein and DNA provide an approach to evaluating potential biomarkers. The present study assessed ethanol-induced oxidative stress by detecting levels of MDA, one of the potential biomarkers of lipid peroxidation. We found that gavage with ethanol at 5 g/kg BW every 24 h for 1 and 4 weeks could induce oxidative stress. The pathogenesis of ethanol-induced oxidative stress has been demonstrated by several studies. Moreover, the present study determined levels of liver enzymes including ALT and AST. Normally, ALT is mainly found in the liver and in smaller amounts in other organs such as the kidneys, pancreas and heart, whereas AST is normally found in the liver, pancreas, red blood cells, heart, muscle and kidneys. The present study demon-

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Fig. 6. Effect of HO-1 on liver histopathology of male Wistar rats (H&E staining) in week 1. (A) Control group. (B) EtOH group. (C) EtOH + ZnPP-IX group. (D) EtOH + Hemin group. The red arrows indicate lipid droplet accumulations in hepatocytes. Scale bar = 20 µm.
strated increased liver enzymes, including ALT and AST, upon ethanol treatment, suggesting ongoing liver damage, similar to previous studies\textsuperscript{19, 20}. The study also showed the increased triglyceride levels in ethanol-treated rats both in weeks 1 and 4, and these findings were related to the gross and microscopic changes of hepatic steatosis. Alcoholic hepatic steatosis is characterized by triglyceride and fatty acid accumulation in hepatocytes, resulting in an increased NADH/NAD\textsuperscript{+} ratio, increased sterol regulatory element-binding protein-1 (SREBP-1) activity, decreased peroxisome proliferator-activated receptor-\(\alpha\) (PPAR-\(\alpha\)) activity and increased complement C3 hepatic levels\textsuperscript{21}. Furthermore, oxidative stress also play the key role in the pathogenesis of alcoholic hepatic steatosis\textsuperscript{14}. The mechanism of alcoholic fatty liver is dependent on the combination of increased glycerolipid synthesis and decreased fatty acid oxidation in mitochondria, which also influence the fat levels\textsuperscript{22}. Several studies have demonstrated chemicals/natural products that ameliorate alcoholic hepatic steatosis\textsuperscript{5, 23, 24}.

Hemin-treated rats showed an increase in HO-1 mRNA expression. HO-1 (32 kDa), also known as heat shock protein-32 (Hsp32), is upregulated by stressors including cytokines, intake of heavy metals, hypoxia and ROS\textsuperscript{25}. It provides defense against oxidative stress by accelerating the degradation of pro-oxidant heme and hemoproteins such as cytochrome P-450 and protoporphyrinogen oxidase and increasing tissue radical scavenging by biliverdin, bilirubin and iron\textsuperscript{25}. We suggest that HO-1 may alleviates alcohol-induced hepatic steatosis by reducing oxidative stress. This study is the first report to demonstrate the protective effect of HO-1 on hepatic steatosis in male Wistar rats in weeks 1 and 4 of ethanol exposure.

In conclusion, alcohol can induce hepatic steatosis through oxidative stress, resulting in elevated levels of liver enzymes, triglyceride and oxidative stress biomarker. This is the first histopathological report demonstrating that HO-1 can alleviate alcoholic hepatic steatosis in male Wistar rats. We suggest that the protective roles of HO-1 may be useful in development of a new therapeutic approach to ameliorate alcoholic hepatic steatosis.

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Fig. 7. Effect of HO-1 on liver histopathology of male Wistar rats (H&E staining) in week 4. (A) Control group. (B) EtOH group. (C) EtOH + ZnPP-IX group. (D) EtOH + Hemin group. The red arrows indicate lipid droplet accumulations in hepatocytes. Scale bar = 20 µm.
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