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## Original Research Article

# Effect of a heme oxygenase-1 inducer on NADPH oxidase expression in alcohol-induced liver injury in male Wistar rats

Phanit Koomhin<sup>1,2</sup>, Chuchard Punsawad<sup>1</sup>, Prasit Suwannalert<sup>3</sup> and Sarawoot Palipoch<sup>1,2\*</sup>

<sup>1</sup>School of Medicine, <sup>2</sup>The Pathobiology of Cell and Tissue Research Group, Walailak University, Nakhon Si Thammarat, 80161, <sup>3</sup>Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

\*For correspondence: **Email:** [spalipoch@hotmail.com](mailto:spalipoch@hotmail.com), [sarawoot.pa@wu.ac.th](mailto:sarawoot.pa@wu.ac.th); **Tel:** +66 7567 2873

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## Abstract

**Purpose:** To investigate the effect of hemin, a heme oxygenase-1 (HO-1) inducer, on nicotinamide adenine dinucleotide phosphate oxidase (NOX) expression in rats with alcohol-induced liver injury.

**Methods:** Male Wistar rats were randomly divided into four groups consisting of the control group, the ethanol (EtOH) group, the EtOH + zinc protoporphyrin IX (ZnPP-IX) group and EtOH + hemin group. Hepatic NOX gene expression and immunohistochemistry of hepatic NOX1 and NOX4 were investigated in week 4.

**Results:** EtOH significantly increased levels of NOX. An immunohistochemical study demonstrated a high number of immunopositive hepatocytes for NOX1 in the EtOH group and EtOH + ZnPP-IX group compared with the control group. Hemin administration downregulated NOX gene expression and lowered the number of immunopositive hepatocytes for NOX1. In contrast, ZnPP-IX (HO-1 inhibitor) administration caused upregulation of NOX gene expression and increased the number of immunopositive hepatocytes for NOX1.

**Conclusion:** HO-1 inducer, hemin, alleviates oxidative stress-induced alcoholic liver injury by reducing NOX, especially NOX1.

**Keywords:** NADPH oxidase, Immunohistochemistry, Heme oxygenase-1, Hemin, Reactive oxygen species, Alcohol-induced liver disease

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## INTRODUCTION

Alcohol-induced liver disease is an increasing global health problem. Acute alcohol consumption leads to fatty liver. Although fatty liver is a reversible injury, its progression can develop into more severe liver problems including steatohepatitis and cirrhosis [1]. Previous studies showed that oxidative stress is an important factor contributing to the development of alcohol-induced liver injury [2]. Ethanol consumption has been shown to

increase the production of reactive oxygen species (ROS)/reactive nitrogen species (RNS), decrease cellular antioxidant levels, and eventually enhance oxidative stress in many tissues, especially the liver [3]. ROS/RNS play the critical roles in damage to several macromolecules especially lipids, proteins and DNA, eventually causing alcohol-induced liver disease [4].

ROS mainly comprise superoxide radical ( $O_2^{\bullet-}$ ), hydroxyl radical ( $\bullet OH$ ), hydrogen peroxide

(H<sub>2</sub>O<sub>2</sub>). A variety of stimuli can produce O<sub>2</sub><sup>•-</sup> through nicotinamide adenine dinucleotide phosphate oxidase (NOX) [5]. NOX1 and NOX4 are expressed in liver; NOX1 mainly produces O<sub>2</sub><sup>•-</sup> whereas NOX4 directly produces H<sub>2</sub>O<sub>2</sub> [6]. NOX1 bind with their structural subunits to form active complexes associated with p22phox, p47phox, p67phox, and active Rac. NOX4 is a constitutively active membrane-bound isoform associated with p22phox only [7]. Oxidative stress can cause increased expression of heme oxygenase-1 (HO-1) levels [8]. Recently, Palipoch et al. demonstrated that HO-1 inducer hemin can relieve alcohol-induced liver injury, especially hepatic steatosis [9]. However, the underlying mechanisms of hemin remain unclear. Accordingly, this study aims to investigate the effect of HO-1 inducer hemin on NADPH oxidase expression in alcohol-induced liver injury in male Wistar rats.

## EXPERIMENTAL

### Animals

Male Wistar rat strains (n=24) (*Rattus norvegicus*) 12 weeks of age were purchased from the Division of Animal House, Faculty of Science, Prince of Songkla University, Thailand. Animal procedures were performed with the approval of the Animal Ethics Committee, Walailak University (protocol no. 003/2014) and followed the Guide for the Care and Use of Laboratory Animals, National Research Council [10]. The animals were provided free access to standard commercial rat diet and distilled water before the experiment.

Rats were randomly divided into four groups (n = 6 per group): 1) rats gavaged with normal saline (control group); 2) rats gavaged with ethanol (30 % v/v in saline, 5 g/kg) once a day for 4 weeks (the EtOH group); 3) rats gavaged with ethanol and intraperitoneally (i.p.) injected with zinc protoporphyrin IX (ZnPP-IX) (20 µmol/kg), a HO-1 inhibitor, every week for 4 weeks (the EtOH + ZnPP-IX group); and 4) rats gavaged with ethanol and i.p. injected with hemin (30 µmol/kg), a HO-1 inducer, every week for 4 weeks (the EtOH + hemin group). Alcohol feeding was performed according to the procedure of Nan et al. [11]. In weeks 4, the rats were euthanized with an anesthetizing thiopental sodium overdose (100 mg/kg BW). The abdominal cavity was then opened, and the liver was collected.

### Hepatic NOX gene expression by RT-PCR

Hepatic total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany). The content

and purity of RNA were measured using a UV spectrophotometer. Reverse transcription-polymerase chain reaction (RT-PCR) was performed for amplification of the NOX gene. The thermal cycling conditions were set up with an initial denaturation step at 95 °C for 15 min and at 94 °C for 1 min. The second step included an annealing of primer at 65 °C for 1 min and the third step comprised an extension at 72 °C for 1 min. The final step comprised an elongation at 72 °C for 10 min. The NOX primers were 5'-GGAAATAGAAAGTT GACTGGCCC-3', forward, and 5'-GTATGAGTGCCATCCAGAGCAG-3', reverse [12], and the beta actin primers were 5'-TTCTTTGCAGCTCCTTCGTTGCCG-3', forward, and 5'-TGGATGGCT ACGTACATGG CTGGG-3', reverse [13]. The DNA samples were loaded into 2 % gel agarose. When stained with ethidium bromide, the gel was visualized with an UV transilluminator. The amount of PCR product was detected using the GeneTools software with image analysis (Syngene, Frederick, MD, USA).

### Immunohistochemistry of hepatic NOX1 and NOX4

The liver sections were de-paraffinized and rehydrated. Heat-induced epitope retrieval was performed in sodium citrate buffer solution, pH 6.0 (Vector Laboratories, CA, USA), using a microwave. Endogenous peroxidase activity was quenched in 3 % hydrogen peroxide in distilled water. Subsequently, the sections were incubated with the blocking buffer (normal goat serum) at room temperature for 30 min for blocking nonspecific binding site. After draining off the blocking buffer, the liver sections were incubated with optimal primary antibody containing rabbit anti-mouse NOX1 (Santa Cruz Biotechnology Inc., USA) and rabbit anti-mouse NOX4 (Santa Cruz Biotechnology Inc., USA) in a humidified chamber at 4 °C overnight.

After washing them three times with phosphate-buffered saline (PBS), the sections were incubated with appropriately diluted secondary antibody (Santa Cruz Biotechnology Inc., USA) in a humidified chamber at room temperature for 30 min. After washing with PBS, avidin-biotin complex (ABC) conjugated with horseradish peroxidase (HRP) (Vectastain ABC Kit, USA) was added to the sections. Subsequently, the sections were washed with PBS, and then the DAB Kit (Vector Laboratories, USA) was applied for 3 min. After counterstaining with Mayer's hematoxylin (Merck, Darmstadt, Germany), the liver sections were dehydrated and mounted [14] and semi-quantitatively examined under a light microscope. All slides were randomly evaluated

in 50 microscopic fields at high magnification by two independent observers (SP and CP).

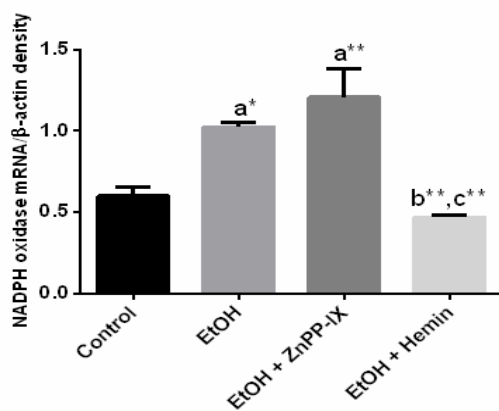
### Data analysis

Statistical analysis was performed using IBM SPSS statistics (version 23). All data are presented as mean  $\pm$  standard error of the mean (SEM). Comparison between groups were performed using one-way analysis of variance (ANOVA). Post hoc comparison was performed using least significant difference (LSD) test. Significance level was set at  $p < 0.05$ .

## RESULTS

### HO-1 inducer hemin reduced NOX mRNA levels in ethanol - treated rats

The rats treated with ethanol (the EtOH group) and the rats treated with ethanol and injected with zinc protoporphyrin IX (the EtOH + ZnPP-IX group) had significantly enhanced ( $p < 0.05$ ) NOX mRNA levels in the liver compared with rats in the control group, as shown in Figure 1. The rats treated with hemin had statistically significantly reduced ( $p < 0.001$ ) NOX mRNA levels compared with rats in the EtOH group and the EtOH + ZnPP-IX group.

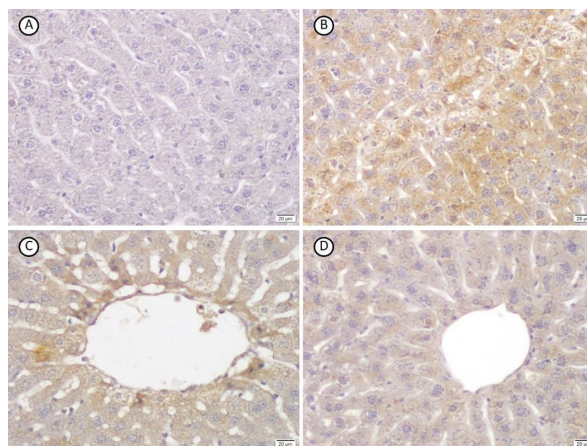


**Figure 1:** Hepatic NOX mRNA levels at week 4 (per group  $n = 6$ ). <sup>a, b, c</sup> Compared with the control group, the EtOH group, and the EtOH + ZnPP-IX group, respectively.  $p < 0.05$ ,  $** p < 0.001$

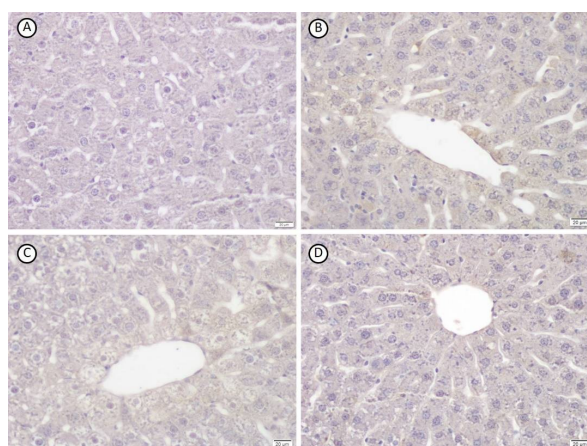
### HO-1 inducer hemin reduced hepatic NOX1

Immunoperoxidase staining for NOX1 and NOX4 in rat hepatocytes at week 4 was illustrated in Figure 2 and Figure 3, respectively. The rats in the EtOH group and EtOH + ZnPP-IX group demonstrated a high number of immunopositive hepatocytes for NOX1 compared with the control group. The rats treated with HO-1 inducer hemin demonstrated a lower number of immunopositive hepatocytes for NOX1 compared to rats in the

EtOH group and EtOH + ZnPP-IX group. In contrast, no difference in immunoreactivity for hepatic NOX4 was demonstrated among the groups.



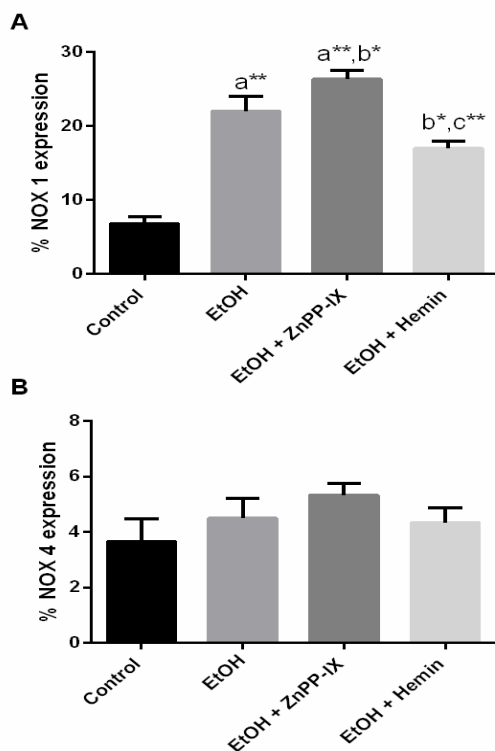
**Figure 2:** Immunoperoxidase staining for NOX1 in rat hepatocytes at week 4. (A) Rats in the control group. (B) Rats treated with ethanol. (C) Rats treated with an HO-1 inhibitor (ZnPP-IX) following ethanol gavage. (D) Rats treated with an HO-1 inducer (hemin) following ethanol gavage. Scale bar = 20  $\mu$ m



**Figure 3:** Immunoperoxidase staining for NOX4 in rat hepatocytes at week 4. (A) Rats in the control group. (B) Rats treated with ethanol. (C) Rats treated with an HO-1 inhibitor (ZnPP-IX) following ethanol gavage. (D) Rats treated with an HO-1 inducer (hemin) following ethanol gavage. Scale bar = 20  $\mu$ m

As shown in Figure 4A, the percentage of positive stained hepatocytes for NOX1 was significantly enhanced ( $p < 0.001$ ) for the ethanol-treated rats compared with rats in the control group. The rats treated with an HO-1 inhibitor (ZnPP-IX) following ethanol gavage had a significantly elevated ( $p < 0.001$ ) percentage of positive stained hepatocytes compared with the ethanol-treated rats and rats in the control group. The rats treated with an HO-1 inducer (hemin) following ethanol gavage had a significantly decreased percentage of positive stained

hepatocytes compared with the ethanol-treated rats ( $p < 0.05$ ) and HO-1 inhibitor-treated rats ( $p < 0.001$ ). No difference in percentage of positive stained hepatocytes for NOX4 for male Wistar rats was demonstrated among the groups (Figure 4B).



**Figure 4:** The percentage of positive stained hepatocytes for NOX1 (A) and NOX4 (B) at week 4 (per group  $n = 6$ ); <sup>a, b, c</sup> compared with control group, the EtOH group and the EtOH + ZnPP-IX group, respectively.  $p < 0.05$ ,  $**p < 0.001$

## DISCUSSION

The liver is a major target organ of alcohol-induced injury. Oxidative stress is believed to involve liver damage induced by alcohol. Increased production of oxidants, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause oxidative stress, which can damage cell components, including DNA, proteins and lipids [15]. Oxidation of ethanol leads to the pathology of liver disease, which comprises three major lesions and includes fatty liver, alcoholic hepatitis and cirrhosis, and eventually causes hepatocellular carcinoma. Research including *in vitro*, *in vivo* and clinical studies was investigated to understand the mechanism behind and develop a new approach to the management of alcohol-induced liver disease. Recently, Palipoch *et al* demonstrated that ethanol treatment of male Wistar rats by gavage for 4 weeks can induce fatty liver, with elevated levels of oxidative stress

biomarker. This indicates oxidative stress is an important mechanism in inducing alcohol-induced liver injury [9].

Alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) are major pathways to oxidize ethanol to acetaldehyde. CYP2E1 is an effective generator of ROS [16]. Various endogenous sources such as inflammatory cells, respiratory chain, and nicotinamide adenine dinucleotide phosphate oxidase (NOX) can produce ROS [24]. Several types of NOX can be found in various cell types. NOX1 and NOX4 are the main ROS-producing NOX in hepatocytes [6]. A previous study demonstrated that CYP2E1 may play an important role in hepatocarcinogenesis induced by ethanol through causing oxidative stress to DNA, while NOX can produce oxidants involved in developing liver injury induced by ethanol [17]. The results of the present study indicate that NOX gene expression was elevated in the ethanol-treated rats, similar to previous studies [18,19]. Moreover, NOX1 was increased in liver sections of ethanol-treated rats detected by immunohistochemistry. We suggest that NOX1 is a key NOX that produces oxidants upon ethanol-induced liver injury.

Normally, oxidants are cleared via an antioxidant defense mechanism. Inefficiency of the antioxidant defense system causes oxidative stress-induced pathologies. Current studies have shown increased interest in investigating effective exogenous antioxidants to ameliorate oxidative stress-related pathologies. Heme oxygenase (HO) is a critical stress-response protein that plays a key role in defense against oxidative stress [19-20]. Three different isoforms of mammalian HO have been reported, including inducible HO-1, constitutive HO-2, and HO-3, which is not catalytically active [15]. HO-1 (heat shock protein 32) plays a potential role in cytoprotective stress and acts as a defensive mechanism against oxidative stress. HO-1 catalyzes the degradation of pro-oxidant heme to radical scavenging bile pigments, biliverdin, and bilirubin [22-24]. The present study demonstrates that an HO-1 inducer inhibits NOX gene expression and reduces NOX1 expression in liver sections. We suggest that an HO-1 inducer alleviates oxidative stress-induced alcoholic liver injury through reducing NOX, especially, NOX1.

## CONCLUSION

Oxidative stress is a major inducer of alcoholic liver injury through increased NOX gene expression and elevated expression of NOX1 in liver sections. An HO-1 inducer, hemin, alleviates



oxidative stress-induced alcoholic liver injury by reducing NOX, especially NOX1. Thus, heme may be useful in developing an effective antioxidant supplementation to ameliorate alcohol-induced liver injury.

## DECLARATIONS

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### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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