

Research Article

Curcumin Decreased Oxidative Stress, Inhibited NF- κ B Activation, and Improved Liver Pathology in Ethanol-Induced Liver Injury in Rats

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To study the mechanism of curcumin-attenuated inflammation and liver pathology in early stage of alcoholic liver disease, female Sprague-Dawley rats were divided into four groups and treated with ethanol or curcumin via an intragastric tube for 4 weeks. A control group treated with distilled water, and an ethanol group was treated with ethanol (7.5 g/kg bw). Treatment groups were fed with ethanol supplemented with curcumin (400 or 1 200 mg/kg bw). The liver histopathology in ethanol group revealed mild-to-moderate steatosis and mild necroinflammation. Hepatic MDA, hepatocyte apoptosis, and NF- κ B activation increased significantly in ethanol-treated group when compared with control. Curcumin treatments resulted in improving of liver pathology, decreasing the elevation of hepatic MDA, and inhibition of NF- κ B activation. The 400 mg/kg bw of curcumin treatment revealed only a trend of decreased hepatocyte apoptosis. However, the results of SOD activity, PPAR γ protein expression showed no difference among the groups. In conclusion, curcumin improved liver histopathology in early stage of ethanol-induced liver injury by reduction of oxidative stress and inhibition of NF- κ B activation.

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1. Introduction

Alcoholic liver disease (ALD) represents a spectrum of clinical illness and morphological changes that range from fatty liver, hepatic inflammation, and necrosis (alcoholic hepatitis) to progressive fibrosis (alcoholic cirrhosis) [1]. Many of the toxic effects of ethanol in the liver have been associated with its metabolism. Ethanol oxidation generates toxic products such as acetaldehyde, and reactive oxygen species result in oxidative stress that initiates apoptosis and cell injury [2–5].

NF- κ B is a transcription factor which regulates genes involving in inflammation. It is activated by endotoxin, cytokines, and oxidative stress [6]. In unstimulated cells, NF- κ B is a heterodimeric complex that is sequestered in the cytoplasm by its interaction with I κ B family of inhibitors.

When these cells are stimulated, I κ B is phosphorylated with subsequent release of NF- κ B resulting in the translocation of NF- κ B from the cytoplasm to the nucleus where it activates the expression of target genes [7, 8]. Activation of NF- κ B increased expression of proinflammatory cytokines and chemokines that were key factors in ethanol-induced liver injury rats [9–12].

Peroxisome proliferators activated receptors gamma (PPAR γ) is a family of ligand-activated nuclear transcriptional factor which regulates cell differentiation, apoptosis, lipid metabolism, and inflammation [13]. More recently, decreased expression of PPAR γ has been found in rats with alcoholic liver fibrosis. These suggested that PPAR γ may play an important role in the development of hepatocellular inflammation, necrosis, and fibrosis in rats with ethanol consumption [14].

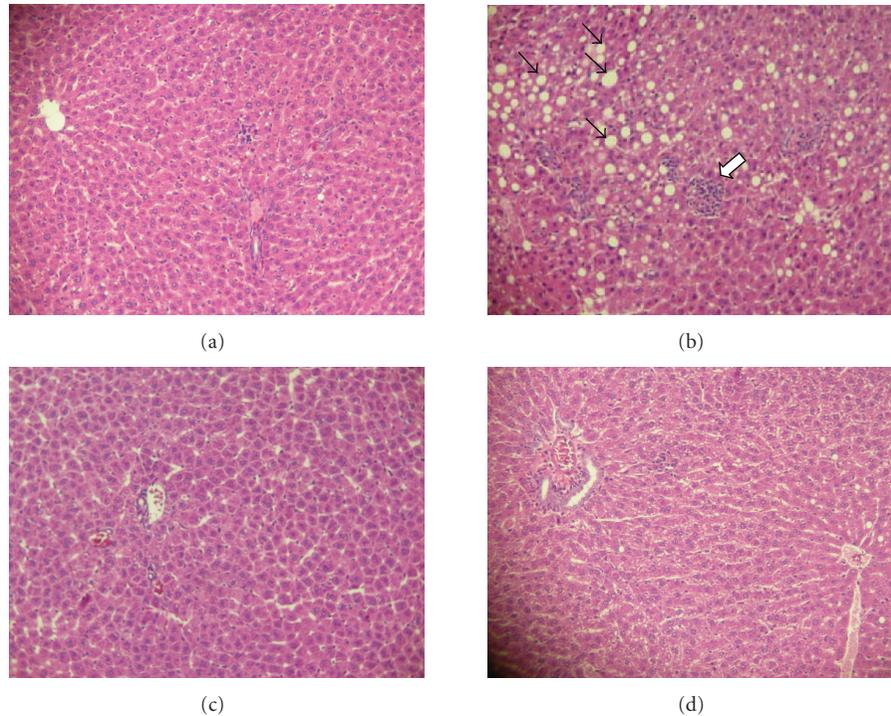


FIGURE 1: Hematoxylin-eosin-stained liver sections (x400). (a) Control group showed normal liver histopathology. (b) Ethanol-treated group showed steatosis (small arrows) and infiltration of inflammatory cells (block arrow). (c) and (d) Curcumin treatment (400 or 1,200 mg/kg bw a day) showed examples of improvement in steatosis and inflammation.

Curcumin (diferuloylmethane), an antiinflammatory and antioxidant compound, is isolated from the rhizomes of the plant *Curcuma longa* Linn. Importantly, it has been shown that curcumin suppressed the activation of NF- κ B in ethanol-induced liver injury in rats [10]. Activation of PPAR γ by curcumin resulted in inhibition of NF- κ B transactivating activity and increased expression of PPAR γ at both the transcriptional and translational levels in activated hepatic stellate cells (HSCs) [15].

However, it is unclear whether curcumin had any effect in early stage of ethanol-induced liver injury. Therefore, the present study determined the effect of curcumin on early stage of ethanol-induced liver inflammation and improved pathology in rats.

2. Materials and Methods

2.1. Animal Preparation. Female Sprague-Dawley rats, weighing 180–220 grams, purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn pathom, were used. The rats were kept in a controlled temperature room at $25 \pm 1^\circ\text{C}$ under standard conditions (12-hour day-night rhythm). All rats were received well care in accordance with the Ethical Committee, Faculty of Medicine, Chulalongkorn University, Thailand.

2.2. Curcumin Preparation. Curcumin in powder form (Cayman Chemical Company, USA) is dissolved in 50% ethanol that freshly prepared for the experiment.

2.3. Experimental Protocol. All rats were fed with the controlled diet which contained 35% of energy from fat, 18% from protein, and 47% from carbohydrate for 4 weeks ad libitum [16]. They were randomly divided into four experimental groups.

Group 1 (Control, $n = 8$): rats were fed distilled water (2.0 mL) orally via an intragastric tube once per day for 4 weeks.

Group 2 (Ethanol, $n = 8$): rats were fed 50% ethanol (7.5 g/kg bw a day) orally via an intragastric tube twice a day for 4 weeks.

Group 3 (Ethanol + curI, $n = 6$): rats were fed curcumin (200 mg/kg bw) dissolved in 50% ethanol (7.5 g/kg bw a day) via intragastric tube twice a day for 4 weeks.

Group 4 (Ethanol + curII, $n = 7$): rats were fed curcumin (600 mg/kg bw) dissolved in 50% ethanol (7.5 g/kg BW a day) by using intragastric tube twice a day for 4 weeks.

At the end of the study, all rats were sacrificed using intraperitoneal injection of an overdose of thiopental sodium. The abdominal walls were opened, and the whole liver was removed. Three small pieces of livers were collected, frozen in liquid nitrogen, and stored at -80°C for MDA analysis, SOD activity, and PPAR γ protein expression. The remaining of liver was fixed in 10% formalin solution to determine histopathology, NF- κ B activation, and hepatic apoptosis.

2.4. Histopathological Examination. After the liver samples were fixed in 10% formalin solution at room temperature,

they were processed by the standard method. Briefly, tissues were embedded in paraffin, sectioned at 5 μm , and stained with Hematoxylin-Eosin, and then picked up on glass slides for light microscopy. All samples were evaluated by an experienced pathologist who is blinded to the experiment. All fields in each section were examined for grading of steatosis and necroinflammation according to Colantoni et al.'s criteria [17].

Steatosis was scored as the percentage of parenchymal cells containing fat (micro- or macrosteatosis):

- 0 = no parenchymal cells containing fat,
- 1 = <20% of parenchymal cells containing fat,
- 2 = 20–39% of parenchymal cells containing fat,
- 3 = 40–50% of parenchymal cells containing fat,
- 4 = >51% of parenchymal cells containing fat.

Inflammation and necrosis were scored by the number of foci of inflammation and necrosis identified under low-power field of light microscope:

- 0 = no inflammation and necrosis,
- 1 = 1 focus per low-power field of inflammation and necrosis,
- 2 = 2 foci per low-power field of inflammation and necrosis,
- 3 = 3 or more foci per low-power field of inflammation and necrosis.

2.5. Hepatic Malondialdehyde (MDA) Determination. MDA was assayed by determining the rate of production of thiobarbituric acid-reactive components [18]. One gram of the liver was homogenized in 1.15% KCl buffer on ice. An aliquot of 0.2 mL was mixed with solution containing 20% acetic acid, 0.8% thiobarbituric acid, and 8.1% sodium dodecyl sulfate, heated in water bath at 95°C for 60 minutes. The solution was centrifuged for 10 minutes at 4000 rpm, and the absorbance of the supernatant fraction was determined at a wavelength of 546 nm. The content of MDA was expressed in terms of nmol/mg protein.

2.6. Hepatic Superoxide Dismutase (SOD) Activity. SOD was determined using the method of Winterbourn, in which the light-triggered release of superoxide radicals from riboflavin leads to the formation of a blue complex through reaction with nitroblue tetrazolium [19]. One gram of the liver was homogenized in 0.1 M phosphate buffer pH 7.4 on ice and cleared by centrifugation at 3000 rpm at 4°C for 15 minutes. The supernatant fraction was incubated in solution containing 0.067 M phosphate buffer pH 7.8, 0.1 M EDTA, 1.5 mM NBT and 0.12 mM riboflavin for 10 minutes in an illuminated chamber with an 18 W fluorescent lamp. Absorbance was recorded at 560 nm, and SOD activity was expressed as units/mg protein.

2.7. Hepatic Apoptosis Determination. Apoptosis was measured by the identification of apoptotic nuclei in sections of

liver by fragment end labeling of DNA (Apoptosis detection kit, Chemicon, USA). In brief, endogenous peroxidase activity was inactivated by 3% hydrogen peroxide (H_2O_2). The DNA fragments were allowed to bind an antidigoxigenin antibody that was conjugated to a peroxidase. Diaminobenzidine (DAB) was applied to develop dark brown color and then the slides were counterstained with hematoxylin. All fields in each sample were evaluated for positive stained liver cell. The results were expressed as the number of positive stained cells per high-power field.

2.8. Immunohistochemistry for Expression of NF- κB p65 in Liver. The liver sections were deparaffinized with xylene and ethanol for ten minutes. After water washing, sections retrieved the antigen (NF- κB p65, Santa Cruz, USA) with citrate buffer pH 6.0 in microwave for thirteen minutes. Next, 3% H_2O_2 and 3% normal horse serum were performed on the slides to block endogenous peroxidase activity for five minutes and blocked nonspecific binding for twenty minutes, respectively. Then, the primary antibody used for NF- κB p65, a polyclonal antibody against the p65 subunit, was applied at a dilution of 1:150 for one hour at room temperature and incubated with the secondary antibody for thirty minutes. When the development of the color with DAB was detected, the slides were counterstained with hematoxylin.

Under light microscopy, the positive stained cells presented dark brown in nucleus. The results were expressed as the number of positive stained cells per high-power field.

2.9. Western Blot Analysis of PPAR γ Protein Expression in Liver. Liver sample (0.1 g) was homogenized in 1 mL of lysis buffer for 30 minutes on ice and cleared by centrifugation at 12000 rpm for 15 minutes at 4°C. Protein concentration was assessed by the Lowry method [20]. A 60 μg of protein was applied to 10% SDS-PAGE gel, and the fractionated proteins were transferred to polyvinylidene fluoride membrane. Membrane was blocked in TBST containing 5% dry nonfat milk for 1 hour and then incubated with PPAR γ monoclonal antibodies (1:400, Santa Cruz, USA) overnight at 4°C. Then washed three times and incubated with secondary antibody, goat antimouse IgG horseradish peroxidase (1:4,000, Cayman, USA) for 1 hour. Protein band was visualized by ECL western blotting system (Amersham, USA). The band densities were normalized by β -actin using a Scion Image system.

2.10. Data Analysis. All data were presented as means and standard deviation (SD). For comparison among all groups of animals, one way analysis of variance (one-way ANOVA) and Tukey posthoc comparisons were employed. Differences were considered statistically significant at $P < .05$.

3. Results

3.1. Histopathological Examination. The histologic appearance of the liver in the control group was normal (Figure 1(a)). In the ethanol-treated group, the histologic

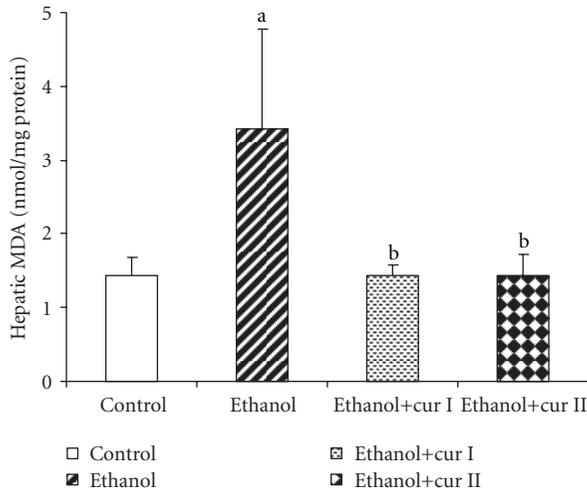


FIGURE 2: Hepatic MDA levels in all groups. All data are expressed as mean \pm SD. The hepatic MDA levels were significantly higher in the ethanol-treated group (Ethanol) when compared with control group (^a $P < .05$). Two doses of curcumin treatment (Ethanol + curI and Ethanol + curII) decreased significantly hepatic MDA levels when compared with Ethanol (^b $P < .05$).

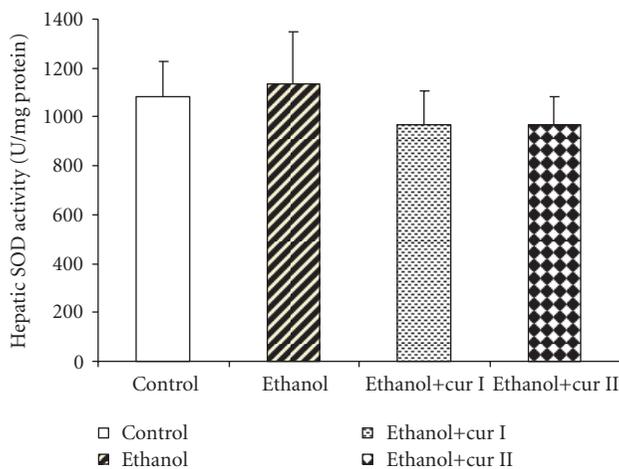


FIGURE 3: Hepatic SOD activity in all groups. All data are expressed as mean \pm SD. No significant differences were observed among groups.

features showed mild to moderate steatosis and mild necroinflammation (Figure 1(b)). Rats treated with ethanol and curcumin 400 mg/kg bw a day improved the liver histopathology that showed only mild steatosis but not necroinflammation (Figure 1(c)). The high dose of curcumin treatment (1,200 mg/kg bw a day) also improved the liver histopathology that showed mild steatosis and mild necroinflammation (Figure 1(d)). The summary of steatosis and necroinflammation score were shown in Table 1.

3.2. Hepatic MDA Level. The level of hepatic MDA, a marker of lipid peroxidation, increased significantly in ethanol-treated group as compared with control group (3.42 ± 1.36

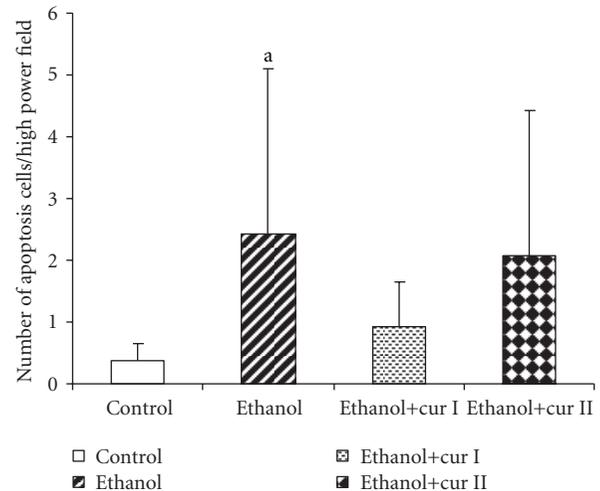


FIGURE 4: Number of apoptosis cells in all groups. All data are expressed as mean \pm SD. Ethanol-treated group (Ethanol) increased significantly apoptotic cells when compared with control group (^a $P < .05$).

TABLE 1: Summary of steatosis and necroinflammation score in all groups. Data are expressed as the number of rats exhibiting the grade of steatosis or necroinflammation indicated.

Group	Number	Steatosis ^a				Necroinflammation ^b			
		0	1	2	3	4	0	1	2
Control	8	8	—	—	—	8	—	—	—
Ethanol	8	—	1	6	1	—	8	—	—
Ethanol + curI	6	5	1	—	—	6	—	—	—
Ethanol + curII	7	4	3	—	—	3	4	—	—

^aThe severity of steatosis was grade by the following. 0 = no parenchymal cells containing fat, 1 = <20% of parenchymal cells containing fat, 2 = 20–39% of parenchymal cells containing fat, 3 = 40–50% of parenchymal cells containing fat, 4 = >51% of parenchymal cells containing fat.

^bThe severity of necroinflammation was grade by the following. 0 = no inflammation and necrosis under low-power field, 1 = 1 focus per low-power field, 2 = 2 foci per low-power field, 3 = 3 or more foci per low-power field.

versus 1.44 ± 0.24 nmol/mg protein, $P < .05$). Curcumin treatment (400 or 1,200 mg/kg bw a day) decreased the elevation of hepatic MDA level significantly when compared with ethanol-treated group (1.43 ± 0.14 versus 3.42 ± 1.36 and 1.43 ± 0.29 versus 3.42 ± 1.36 nmol/mg protein, resp.; $P < .05$) (Figure 2).

3.3. Hepatic SOD Activity. SOD enzyme converts O_2^- into a less toxic product. This enzyme is the first line in cell defense against oxidative stress. Our results showed that the level of hepatic SOD activity of the control group was 1081.36 ± 145.01 units/mg protein, while that of the ethanol-treated group was 1135.86 ± 209.48 units/mg protein. In rats treated with ethanol and curcumin (400 or 1,200 mg/kg bw a day), the levels of hepatic SOD activity were 966.28 ± 139.44 and 967.84 ± 116.66 units/mg protein, respectively. There was no significant difference among groups (Figure 3).

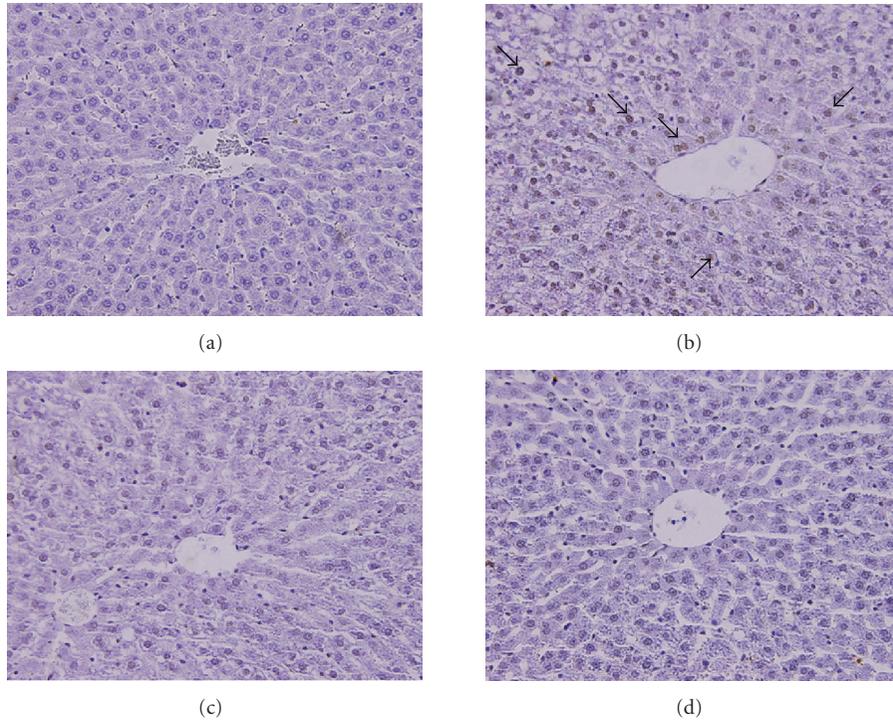


FIGURE 5: Representative liver sections processed for apoptosis assay by TUNEL reaction (x400). (a) Control group. (b) Ethanol-treated group, the black arrows indicated a TUNEL-positive apoptotic hepatocyte found frequently around central vein. (c) and (d) curcumin treatment (400 or 1,200 mg/kg bw a day) showed a decrease of apoptotic hepatocytes.

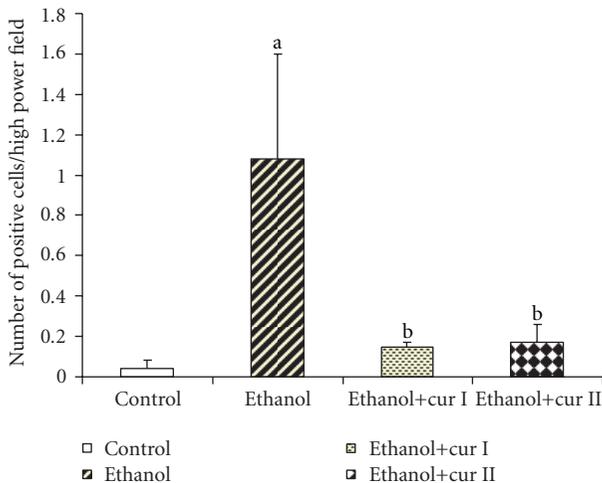


FIGURE 6: Number of NF-κB p65 positive cells/high-power field in all groups. All data are expressed as mean ± SD. Ethanol-treated group (Ethanol) increased significantly NF-κB p65 positive cells when compared with control group (^a $P < .05$). Two doses of curcumin treatment (Ethanol + curI and Ethanol + curII) decreased significantly NF-κB p65 positive cells when compared with Ethanol (^b $P < .05$).

3.4. Hepatic Apoptosis. Hepatocyte apoptosis was determined by TUNEL assay. The number of apoptotic nuclei in the liver of control group was very low (0.38 ± 0.28 cells/high-power field). In contrast, the numbers of apoptotic cells were

observed frequently in centrilobular area in ethanol-treated group when compared with control group (2.43 ± 2.68 versus 0.38 ± 0.28 cells/high-power field, $P < .05$) (Figures 4 and 5). There was a trend of decreased apoptosis in low dose of curcumin treatment, but the difference did not reach a statistical significance (Figure 4).

3.5. Expression of NF-κB p65 in Liver. The expression of NF-κB p65 in liver was determined by immunohistochemistry. The data of NF-κB p65 expression in all groups were given in Figure 6. The number of positive stained cells in the liver of ethanol-treated group was significantly higher than control group (1.08 ± 0.52 versus 0.04 ± 0.04 cells/high-power field, $P < .05$). In contrast, curcumin treatment (400 or 1,200 mg/kg bw a day) decreased the number of positive stained cells significantly when compared with ethanol-treated group (0.15 ± 0.02 versus 1.08 ± 0.52 and 0.17 ± 0.09 versus 1.08 ± 0.52 cells/high-power field, resp.; $P < .05$) (Figures 6 and 7).

3.6. PPARγ Protein Expression in Liver. In order to examine the change of PPARγ protein expression in early stage of ethanol-induced liver injury, we measured PPARγ protein expression in the liver. The PPARγ protein expression in control group was 0.57 ± 0.19 , and ethanol group was 0.68 ± 0.16 . Rats treated with ethanol and curcumin (400 or 1,200 mg/kg bw a day) had 0.44 ± 0.03 and 0.54 ± 0.23 ,

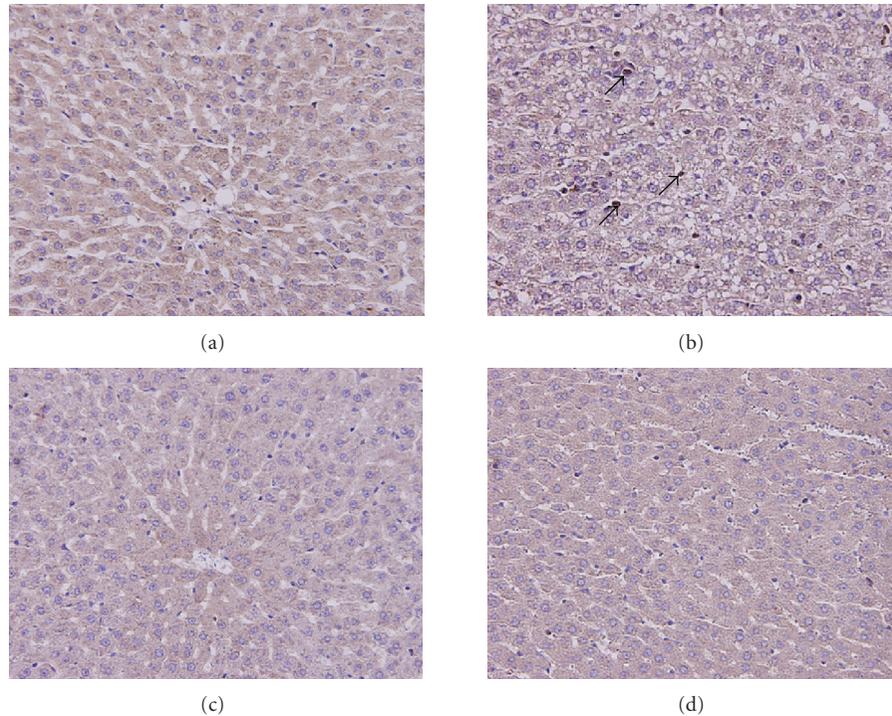


FIGURE 7: Immunohistochemistry of NF- κ B p65 expression in rat liver (x400). (a) Control group. (b) Ethanol-treated group showed NF- κ B p65 positive cells in nuclei (black arrow). (c) and (d) curcumin treatment (400 or 1,200 mg/kg bw a day) showed a diminishment of NF- κ B expression in hepatocyte nuclei.

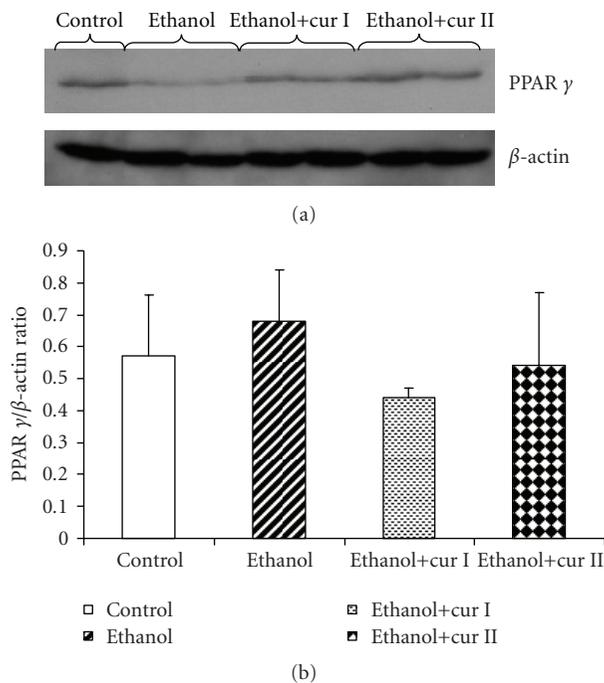


FIGURE 8: PPAR γ protein expression determined by Western blot analysis. (a) represented bands of PPAR γ and β -actin. (b) normalized densitometric ratio of PPAR γ to β -actin.

respectively. These data did not show a significant change in PPAR γ protein expression in the liver in all groups (Figure 8).

4. Discussion

Ethanol oxidation generates toxic metabolites, free radicals; and induces a state of oxidative stress which contributes to the pathogenesis of ALD. Importantly, oxidation of ethanol through the cytochrome P450 2E1 (CYP 2E1) generates superoxide anion radical and hydrogen peroxide [2, 21, 22]. These free radicals are capable of damaging many cellular components such as DNA, protein, and lipid [23]. One of the characteristic features of oxidative stress is enhancement of lipid peroxidation. A number of studies have been demonstrated that ethanol intake increased the formation of lipid peroxidation product, such as MDA [24–26]. We found that an increase in hepatic MDA level as well as pathological changes were observed in ethanol-treated group.

To counteract this oxidative stress, cells have a variety of antioxidant enzymes, including SOD, catalase, and glutathione peroxidase. SOD catalyzes the rapid removal of superoxide radicals [27]. The effects of chronic ethanol exposure on activity of SOD are controversial, with reports of decrease or no changes [25, 28]. These studies may reflect variations in experimental design, diet, and duration of ethanol feeding. Decreased SOD activity in ethanol fed rats was associated with enhancement of lipid peroxidation and severe pathology of liver [25]. Our model showed mild histopathology changes in both steatosis and necroinflammation. Therefore, the SOD activity in liver did not change in early ethanol-induced liver injury.

Oxidative stress can also initiate or amplify inflammation through upregulation of several genes involved in the inflammatory response. One such gene is NF- κ B, whose activation results in the upregulation of proinflammatory cytokines [6]. Activation of NF- κ B and upregulation of cytokine production occurred in ethanol-induced liver injury and are associated with lipid peroxidation [9, 10]. Our study confirmed induction of NF- κ B activation in ethanol-treated group. Curcumin is known as antioxidant and antiinflammatory properties. It is the free radical scavenger and inhibited lipid peroxidation product [29–32]. Evidence was presented that curcumin prevented ethanol-induced liver injury in rats by inhibiting the expression of NF- κ B-dependent genes [10]. Although, a high dose of curcumin treatment (1,200 mg/kg bw) was not better than low dose (400 mg/kg bw), the present study showed that curcumin improved ethanol-induced liver injury by reduction of oxidative stress and inhibition of NF- κ B activation.

Ethanol-induced liver injury has been linked to oxidative stress caused by the production of reactive oxygen intermediates that cause mitochondrial dysfunction, leading to a release of proapoptotic factors such as cytochrome c that can activate caspases and initiate the apoptotic cascade in hepatocytes [33]. Jin and coworker observed the pathological changes and investigated the correlation of hepatocyte apoptosis with CYP2E1 expression and oxygen free radical in rats with ALD [34]. Using the TUNEL assay, we detected a difference in apoptosis between the control and ethanol-treated group that was similar to human alcoholic hepatitis and experimental rat model of ALD [33–35]. Cells in centrilobular area are low O₂ and nutrient supply thus the distribution of apoptotic cells is observed frequently in centrilobular area [36]. In this study curcumin treatment did not detect a difference in hepatocyte apoptosis; however, this was a trend of decreased apoptosis in low doses of curcumin treatment.

More recently, decreasing of PPAR γ expression was found in alcoholic liver fibrosis rats [14]. This stage showed severe liver injury and HSC activation. In normal liver, HSCs undergo a process known as activation, which upregulate cytokines and growth factor. For instance, platelet-derived growth factor is capable of inhibiting PPAR γ expression via mitogen-activated protein kinase-mediated phosphorylation of PPAR γ [37]. Also, TNF- α , inflammatory cytokine, is known to inhibit PPAR γ expression in adipocytes and an early phase of HSC activation in liver fibrosis [38, 39], thus alcoholic liver fibrosis rats could decrease PPAR γ expression. Our model showed only mild steatosis, necroinflammation, and no HSC activation; therefore, no change of PPAR γ protein expression was found in ethanol-treated group. Further studies should be determined roles of PPAR γ in different stages of ALD.

In conclusion, our study demonstrated that curcumin, a representative phenolic antioxidant and antiinflammation, could improve histopathology of liver in early stage of ethanol-induced liver injury by reduction of oxidative stress and inhibition of NF- κ B activation. For hepatocyte apoptosis, curcumin treatment might have a trend of decreased apoptotic cells in ethanol-fed rats.

Acknowledgments

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