Original Article

Effects of Curcumin Attenuated Hepatitis in Mice with Paracetamol Overdose

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ABSTRACT

Background: N-acetyl-*p*-aminophenol (APAP) or paracetamol overdose causes increasing of toxic metabolites, which disrupting hepatocyte function, and liver injury occurs. Curcumin has been used for treatment of inflammatory conditions such as hepatitis. Therefore, this study aims to determine effects of curcumin attenuate hepatitis in mice with APAP overdose.

Methods: Male mice (25-30 gram) were divided into four groups. Group I (control); mice were gavaged with distilled water. Group II (APAP); mice were gavaged with a single dose of 400 mg/kg of APAP. Group III (APAP + CUR 200); mice were gavaged with a single dose of 400 mg/kg of APAP and 200 mg/kg of curcumin. Group IV (APAP + CUR 600); mice were gavaged with a single dose of 400 mg/kg of APAP and 600 mg/kg of curcumin. The serum was collected to determine liver enzymes and liver tissues for hepatic MDA, hepatic GSH and histopathology.

Results: Serum ALT, AST and hepatic MDA were significantly increased in APAP when compared with control and significantly decreased in APAP + CUR 200 and APAP + CUR 600 when compared with APAP. Hepatic GSH was significantly decreased in APAP when compared with control and significantly increased in APAP + CUR 200 and APAP + CUR 600 when compared with APAP. Histopathology of APAP showed acute centrilobular hemorrhagic hepatic necrosis involving all zones and the improvement of liver pathology revealed in APAP + CUR 200 and APAP + CUR 600.

Conclusion: These results suggest that APAP overdose is related to liver toxicity. Our results show curcumin can prevent the damage by induction of hepatic GSH, reduction of oxidative stress, attenuation of liver inflammation, and the improvement of liver pathology. In addition, curcumin at the dose of 600 mg/kg tends to be more potent than 200 mg/kg.

Key words : paracetamol, curcumin, hepatitis, oxidative stress

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Drug-induced liver injury (DILI) is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies[1]. According to the United States Acute Liver Failure Study Group, DILI accounts for more than 50% of acute liver failure, including hepatotoxicity caused by overdose of N-acetyl-p-aminophenol (APAP) or paracetamol (39%) and idiosyncratic liver injury triggered by other drugs (13%)[2]. Although the exact mechanism of DILI remains largely unknown, it appears to involve 2 pathways: direct hepatotoxicity and adverse immune reactions. In most instances, DILI is initiated by the bioactivation of drugs to chemically reactive metabolites, which have the ability to interact with cellular macromolecules such as proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress. Additionally, these reactive metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. This impairment of cellular function can culminate in cell death and possible liver failure[3].

Curcumin is the main yellow bioactive component of turmeric *(Curcuma longa Linn.)*. It has been shown to possess a wide spectrum of biological actions. These include anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, and antidiabetic activities[4-6]. The hepatoprotection of curcumin have been widely acknowledged and used in traditional medicine for treatment of inflammatory conditions such as hepatitis[7].

Therefore, this study aims to determine effects of curcumin attenuate hepatitis in mice with APAP overdose.

MATERIALS AND METHODS

Chemicals

Curcumin in powder form (Cayman Chemical Company, USA) was dissolved in corn oil that was freshly prepared for the experiment. A single dose of 200 and 600 mg/kg of curcumin were administered to mice by oral gavage.

A single dose of 400 mg/kg of APAP also known as Tylenol® which is freshly prepared by dissolving in distilled water (dH_2O) and was administered to mice by oral gavage.

Animals

Male mice (4-5 weeks of age), weighing 25-30 gram (g), were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand and use as experimental animals. The mice were acclimatized at least 1 week in a climate-controlled room on a 12-hour (h) light-dark cycle and were fed *ad libitum*. The study was performed in adherence to the National Institutes of Health guidelines for the experimental use of animals and followed a protocol approved by the Animal Care and Use Committee, Faculty of Medicine, Chulalongkorn University, Thailand. The mice were then fasted for 16 h before experiments to sensitize mice to APAP toxicity.

Mice were divided into four groups

Group I (control); mice were gavaged with dH_2O.

Group II (APAP); mice were gavaged with a single dose of 400 mg/kg of APAP.

Group III (APAP + CUR 200); mice were gavaged with a single dose of 400 mg/kg of APAP with a single dose of 200 mg/kg of curcumin.

Group IV (APAP + CUR 600); mice were gavaged with a single dose of 400 mg/kg of APAP with a single dose of 600 mg/kg of curcumin.

Study design

Twenty-four hours after APAP administration, all mice was anesthetized with intraperitoneal (i.p.) injection of thiopental sodium. The abdomen was opened medially and the whole liver was rapidly removed and washed with cold normal saline (4-8˚C). The tissues were chopped into small pieces with scissors, frozen in liquid nitrogen, and stored at -80˚C for hepatic malondialdehyde (MDA) and hepatic glutathione (GSH). The remaining liver was fixed in 10% formalin solution for histopathology. Subsequently, the whole blood of mice was collected from the heart. The blood was allowed to coagulate at room temperature for 2 h and then centrifuged for 20 minutes at 3000 revolution per minute (r.p.m.) to obtain serum. The serum was collected to determine liver enzymes including AST and ALT.

Hepatic MDA assay

Lipid peroxidation of mice liver using thiobarbituric acid (TBA) was measured by a modified method of Ohkawa *et al*[8]. One gram of mice liver tissue was homogenized in 3 mL of 50 mM potassium phosphate buffer (pH 7.0). To 0.3 mL of liver homogenated in test tube, 1.5 mL of 10% trichloroacetic acid (TCA) solution and 1.5 mL of 0.8% TBA solution were added. The mixture was boiled in waterbath at 95˚C for 60 min and then cooling with tap water at room temperature. After centrifugation at 3000 r.p.m. for 15 min, the absorbance of sample was measured at 532 nm. 1, 1, 3, 3 tetramethoxy propane (TMP) was used as a standard of MDA. The MDA content was calculated in comparison with a standard MDA curve and was expressed as nmol/mg protein.

Hepatic GSH assay

Cayman' GSH Assay utilizes a carefully optimized enzymatic recycling method, using GSH reductase, for the quantification of GSH[9]. The sulfhydryl group of GSH reacts with 5, 5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), or Ellman's reagent and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by GSH reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. The optical density (O.D.) of TNB is then measured at 405-414 nm using a microplate reader, which provides an accurate estimation of GSH in the sample.

Histopathology

After the liver samples have been fixed in 10% formalin solution at room temperature, they were processed using standard method. Briefly, tissues were embedded in paraffin, sectioned at 5 μm, and stained with Hematoxylin & Eosin (H & E), and then picked up on glass slides. The histological slides were evaluated under light microscope (LM) by an experienced pathologist who is blinded to the experiment. All fields in each section were examined for grading of hepatic necroinflammation according to the criteria described by Brunt EM *et al*[10] from 0 to 3 as follow; score 0 = no hepatocyte injury/inflammation, score 1= sparse or mild focal zone 3 hepatocyte injury/inflammation, score 2 = noticeable zone 3 hepatocyte injury/inflammation, and score 3 = severe zone 3 hepatocyte injury/inflammation.

Statistical analysis

All data were presented as mean ± 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 $p < 0.05$. The data were analyzed using the SPSS software version 17.0 for Windows.

RESULTS

The serum AST and ALT enzymes were significantly increased in APAP group when compared with control group (AST; 583.25 ± 118.30 vs. 86.13 ± 6.90 U/L and ALT; 186.00 ± 43.73 vs. 42.63 ± 6.95 U/L, $p < 0.05$) and significantly decreased in APAP + CUR 200 (AST; 197.38 ± 14.39 vs. 538.25 ± 118.30 U/L and ALT; 65.25 ± 3.11 vs. 186.00 ± 43.73 U/L, $p < 0.05$) and APAP + CUR 600 groups (AST; 111.38 ± 8.33 vs. 583.25 ± 118.30 U/L and ALT; 47.50 ± 4.72 vs. 186.00 ± 43.73 U/L, $p < 0.05$) when compared with APAP group (Table 1, Figure 1 and 2).

Hepatic GSH was significantly decreased in APAP group when compared with control group (2.75 ± 0.16 vs. 10.17 ± 0.11 nmol/mg protein, $p < 0.05$). These were significantly increased in APAP + CUR 200 (9.16 ± 0.49 vs. 2.75 ± 0.16 nmol/mg protein, $p < 0.05$) and APAP + CUR 600 groups (9.72 ± 0.22 vs. 2.75 ± 0.16 nmol/mg protein, $p < 0.05$) when compared with APAP

Table 1. Summary of parameters in all groups (n = 8 each)

Group	Parameters			
	AST (U/L)	ALT (U/L)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
Control	86.13 ± 6.90	42.63 ± 6.95	10.17 ± 0.11	1.45 ± 0.01
APAP	583.25 ± 118.30[a]	186.00 ± 43.73[a]	2.75 ± 0.16[a]	3.55 ± 0.05[a]
APAP + CUR 200	197.38 ± 14.39[a,b]	65.25 ± 3.11[b]	9.16 ± 0.49[a,b]	1.47 ± 0.01[b]
APAP + CUR 600	111.38 ± 8.33[b,c]	47.50 ± 4.72[b]	9.72 ± 0.22[a,b,c]	1.46 ± 0.01[b]

The results are means ± SD.
[a]$p < 0.05$ compare with control group, [b]$p < 0.05$ compare with APAP group, and [c]$p < 0.05$, compare with APAP + CUR 200 group.

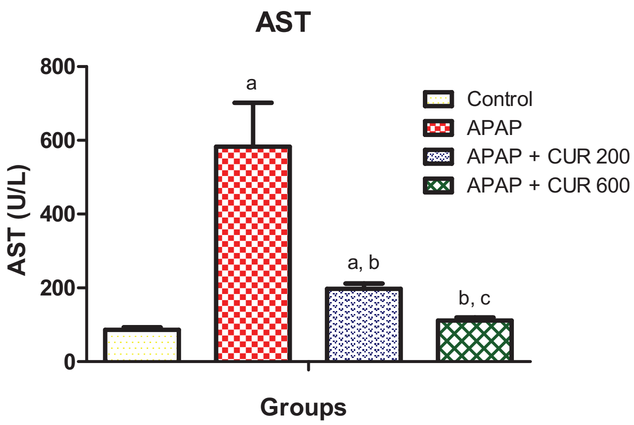

Figure 1. Hepatic enzyme (AST) in serum was determined. $^{a}p < 0.05$ compare with control group, $^{b}p < 0.05$ compare with APAP group, and $^{c}p < 0.05$, compare with APAP + CUR 200 group.

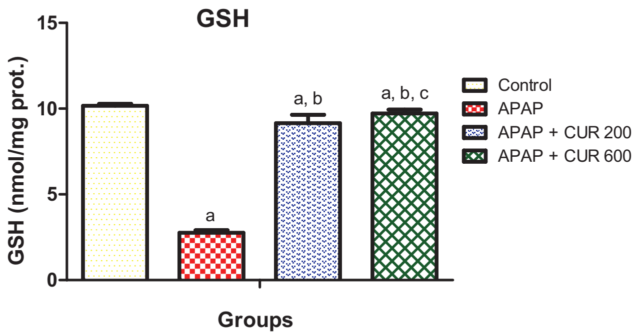

Figure 3. Hepatic GSH was determined. $^{a}p < 0.05$ compare with control group, $^{b}p < 0.05$ compare with APAP group, and $^{c}p < 0.05$, compare with APAP + CUR 200 group.

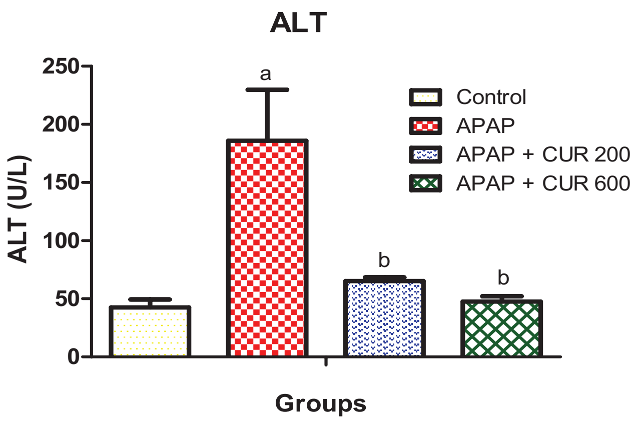

Figure 2. Hepatic enzyme (ALT) in serum was determined. $^{a}p < 0.05$ compare with control group, $^{b}p < 0.05$ compare with APAP group.

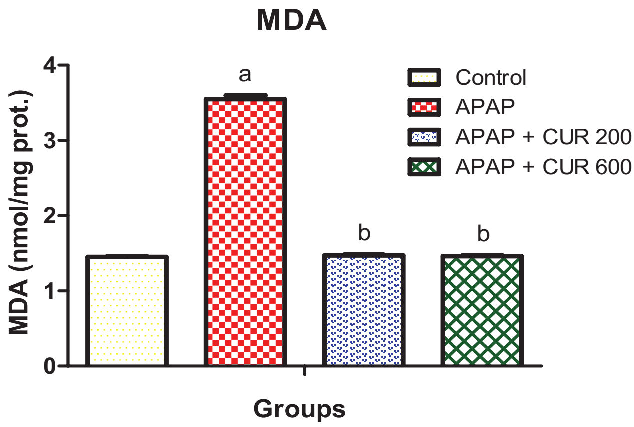

Figure 4. Hepatic MDA in liver homogenate was determined. $^{a}p < 0.05$ compare with control group, $^{b}p < 0.05$ compare with APAP group.

Table 2. Summary of hepatic necroinflammation score in all groups. Data are expressed as the number of mice exhibiting the grade of hepatic necroinflammation indicated.

Group	Number	Hepatic necroinflammation*			
		0 = none	1 = mild	2 = moderate	3 = severe
Control	8	8	-	-	-
APAP	8	-	1	1	6
APAP + CUR 200	8	5	2	1	-
APAP + CUR 600	8	5	3	-	-

*All fields in each section were examined for grading of hepatic necroinflammation according to the criteria described by Brunt EM *et al*[10].
Score 0 = no hepatocyte injury/inflammation
Score 1 = sparse or mild focal zone 3 hepatocyte injury/inflammation
Score 2 = noticeable zone 3 hepatocyte injury/inflammation
Score 3 = severe zone 3 hepatocyte injury/inflammation

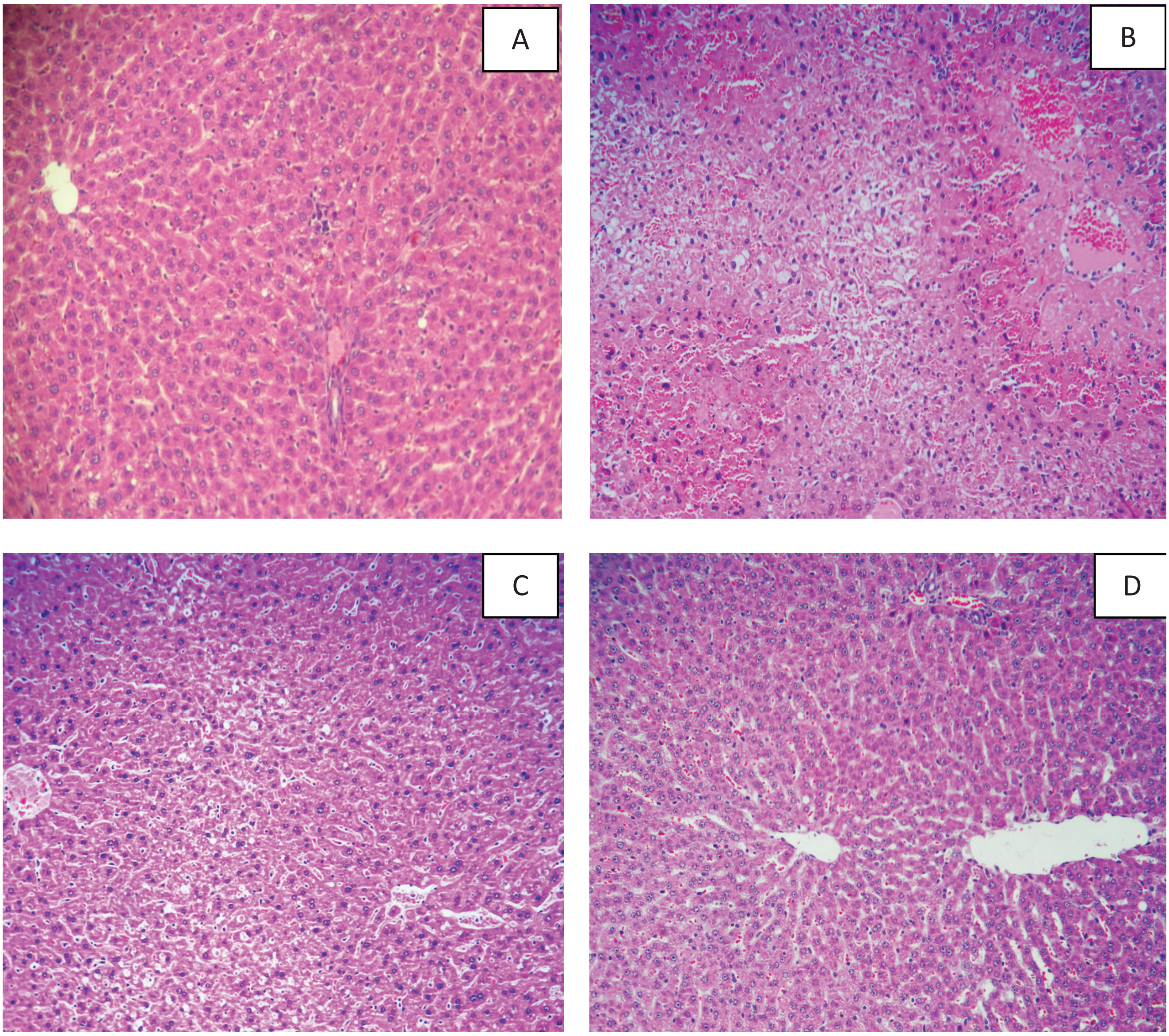

Figure 5. Liver histopathology of H & E staining (A); Control group showed normal hepatic architecture, (B); APAP group showed acute centrilobular hemorrhagic hepatic necrosis involving all zones. (C); APAP + CUR 200 group showed mild focal necrosis with mild fatty changes and the hepatic architecture was preserved, and (D); APAP +CUR 600 group showed the majority of hepatic lobules preserved the normal architecture with limited hepatic change.

group (Table 1 and Figure 3).

Hepatic MDA, a marker of oxidative stress, was significantly increased in APAP group when compared with control group (3.55 ± 0.05 vs. 1.45 ± 0.01 nmol/mg protein, $p < 0.05$). These were significantly decreased in APAP + CUR 200 (1.47 ± 0.01 vs. 3.55 ± 0.05 nmol/mg protein, $p < 0.05$) and APAP + CUR 600 groups (1.46 ± 0.01 vs. 3.55 ± 0.05 nmol/mg protein, $p < 0.05$) when compared with APAP group (Table 1 and Figure 4).

Liver histology in control group showed normal. In APAP group, the hepatic necroinflammation was moderate to severe injury. In APAP + CUR 200 and APAP + CUR 600 groups improved the severity, the hepatic necroinflammation showed mild injury (Table 2 and Figure 5).

Discussion

In therapeutic dose, APAP is mainly metabolized via glucuronidation and sulfation and in conjugated forms is excreted from the body. Besides, APAP partly is metabolized by cytochrome P450 (CYP 2E1), to produce metabolites, mainly NAPQI, which are dramatically increased in high APAP concentrations; these metabolites of APAP are detoxified by GSH and removed from the body. Then, in APAP overdose causes increasing of toxic metabolites. These metabolites interact with a range of cellular proteins via covalent binding, which disrupting hepatocyte functions causing necrosis and liver injury occurs[11,12].

The present study demonstrates that in vivo treatment of male mice with APAP overdose results in hepatic GSH depletion. This result corresponds to previous observations studied in APAP model showing that APAP-induced hepatic GSH depletion[13-15]. GSH serves a nucleophilic co-substrate to glutathione transferase in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides[16,17]. GSH is also involved in amino acid transport and maintenance of protein sulfhydryl reduction status[18,19]. These findings suggest that induction of cytochrome P450 (CYP2E1) in the liver should enhance hepatic GSH depletion.

GSH is essential for conjugation or detoxification of toxic metabolites, mainly NAPQI, which disrupting hepatocyte in APAP-treated mice. GSH (reduced form) is easily oxidized to the disulfide dimer GSSG (oxidized form). GSSH is produced during the reduction of hydroperoxides by glutathione peroxidase enzyme. GSSG is reduced to GSH by glutathione reductase enzyme and it is the reduced form that exists mainly in conjugation or detoxification of toxic metabolites. These findings suggest that curcumin may possibly inducer via glutathione reductase enzyme to produce GSH or inhibitor via glutathione peroxidase enzyme. In addition, curcumin was also found to be a weak inhibitor of cytochrome P450 (CYP 2E1)[20].

The present study corresponds to previous observations studied in APAP model showing that APAP-induced hepatic MDA[13-15]. MDA is the most widely used test for measuring the extent of lipid peroxidation. Lipid peroxidation is a chain reaction process that involves the participation and production of free radical species. Free radicals can cause cellular injury when produced in sufficient amounts to overcome the normally efficient protective mechanism. Lipid peroxidation is a free radical mediated chain reaction which is enhanced as a consequence of oxidative stress (a general term used to describe a stable of damage caused by reactive oxygen species (ROS), and it results in an oxidative deterioration of membrane polyunsaturated fatty acids). It is a continuous physiological process occurring in cell membranes[21], which defined as the oxidative deterioration of polyunsaturated fats. The cell membranes contains large amount of polyunsaturated fatty acids which are especially susceptible to lipid peroxidation. Lipid peroxidation of cell membranes results in decreased membrane fluidity, inability to maintain ionic gradients, cellular swelling, and tissue inflammation. These findings suggest that induction of cytochrome P450 (CYP2E1) in the liver should enhance hepatic MDA production.

The induction of hepatic MDA is essential for lipid peroxidation (oxidative stress), which disrupting hepatocyte in APAP-treated mice. The cell membranes contains large amount of polyunsaturated fatty acids which are especially susceptible to lipid peroxidation. Lipid peroxidation of cell membranes results in cellular swelling and tissue inflammation[21]. These findings suggest that curcumin could be decreased hepatic MDA mediated via covalently bound to cellular macromolecules inhibition.

A previous study demonstrated that curcumin at the dose of 200 mg/kg and 600 mg/kg had an antioxidant and anti-inflammatory property[7]. In this study, 200 mg/kg of curcumin was a sufficient dose for attenuate hepatitis. This finding corresponded to previous observations studied in APAP model showing that APAP-induced hepatitis was attenuated by curcumin[13-15].

Conclusions

In conclusion, these results suggest that APAP toxicity to liver is related to depletion of hepatic GSH concomitant with the induction of oxidative stress, liver inflammation, and the damage of liver pathology. Our results show curcumin can prevent most of the damage caused by APAP overdose in mice by induction of hepatic GSH, reduction of oxidative stress, attenuation of liver inflammation, and the improvement of liver pathology. In addition, curcumin at the dose of 600 mg/kg tends to be more potent than 200 mg/kg in preventing the effects of APAP toxicity. Hence, curcumin might be a new natural therapeutic agent against hepatitis induced by APAP.

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