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Hepatoprotective Effect of Kaempferol Against Alcoholic Liver Injury in Mice

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Abstract: Kaempferol is a biologically active component present in various plants. The hepatoprotective effect of kaempferol in drug-induced liver injury has been proven, while its effect against alcoholic liver injury (ALI) remains unclear. Hence, the present study aimed to evaluate the effect of kaempferol against ALI in mice. The experimental ALI mice model was developed and the mice were treated with different doses of kaempferol for 4 weeks. The liver functions were observed by monitoring the following parameters: Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) levels in serum; histopathological studies of liver tissue; oxidative stress by hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) and glutathione (GSH); the lipid peroxidation status by malondialdehyde (MDA) and lipid accumulation by triglyceride (TG) level in serum; and the expression levels and activities of a key microsomal enzyme cytochrome 2E1 (CYP2E1), by both *in vitro* and *in vivo* methods. The ALI mice (untreated) showed clear symptoms of liver injury, such as significantly increased levels of oxidative stress, lipid peroxidation and excessive CYP2E1 expression and activity. The mice treated with different kaempferol dosages exhibited a significant decrease in the oxidative stress as well as lipid peroxidation, and increased anti-oxidative defense activity. The kaempferol treatment has significantly reduced the expression level and activity of hepatic CYP2E1, thus indicating that kaempferol could down regulate CYP2E1. These findings show the hepatoprotective properties of kaempferol against alcohol-induced liver injury by attenuating the activity and expression of CYP2E1 and by enhancing the protective role of anti-oxidative defense system.

Keywords: Alcoholic Liver Injury (ALI); Kaempferol; Cytochrome 2E1; Hepatoprotective Effect; Oxidative Stress.

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Introduction

Chronic and binge consumption of alcohol results in alcoholic liver injury (ALI). It is one of the major global health problems accounting for approximately 4% of total deaths worldwide (O'Shea *et al.*, 2010; Altamirano and Bataller, 2011).

High alcohol consumption is associated with oxidative stress, which leads to hepatotoxicity by promoting the generation of reactive oxygen species (ROS) and decreasing the anti-oxidant defense (Wang and Cederbaum, 2007). It has been hypothesized that oxidative stress and lipid peroxidation could play important roles in alcohol-induced liver toxicity. Ethanol can be metabolized into ROS by alcohol dehydrogenase (ADH) and cytochrome P450 (CYP450). ROS are released abundantly and triggers toxic effects either directly or indirectly through lipid-peroxides (Cederbaum *et al.*, 2009). The involvement of CYP450 in alcohol metabolism was first identified by Charles S. Lieber in his studies on the microsomal ethanol-oxidizing system (MEOS) (Lieber, 1997). The activity of MEOS increases in chronic alcohol consumption partly due to the induction of CYP450 enzymes. Among the CYP450 family, cytochrome 2E1 (CYP2E1) has been identified as a key microsomal enzyme in ALI, since it is highly inducible with high catalytic activity against alcohol. Several studies have demonstrated that CYP2E1 plays a major role in alcohol-induced liver injury (French, 2013). Recent work has shown that CYP2E1 activity correlates with ethanol-induced liver injury and lipid peroxidation (Bell *et al.*, 2010). Inhibition of CYP2E1 effectively blocked the alcohol-mediated lipid peroxidation and reduced liver injury. In contrast, oxidant stress was exacerbated in both Cyp2e1-knockin mice and human CYP2E1-knockin HepG2 cells (Bai and Cederbaum, 2006). The liver injury caused by oxidative stress and lipid peroxidation can be decreased by knocking out CYP2E1 or inhibiting alcohol-mediated CYP2E1 activation. It has been reported that there are some CYP2E1 inhibitors, but they cannot protect against liver injury effectively (Martikainen *et al.*, 2012). Endogenous GSH level is a major factor in alcohol-induced oxidant stress, and CYP2E1 overexpression in HepG2 cells induces GSH synthesis through the transcriptional activation of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis (Mari and Cederbaum, 2000).

Kaempferol (3,4′,5,7-tetrahydroxyflavone) as a representative flavonoid, is widely distributed in many medicinal herbs, vegetables and fruits. Plants consisting of kaempferol have been suggested as healthy functional food with hepatoprotective activity. Kaempferol has been reported as a substrate and inhibitor of CYP2E1 expression along with anti-oxidant, anti-inflammatory and immunomodulatory properties both *in vitro* and *in vivo* (Martino *et al.*, 2014). Kaempferol has been newly used as an adjuvant for preventing CYP2E1-mediated hepatotoxicity induced by drugs such as INH and RIF (Shih *et al.*, 2013). Additionally, kaempferol has phenolic ring and hydroxyl groups, which are prone to donating a hydrogen atom or an electron to free radicals and the extended conjugated aromatic system can delocalize an unpaired electron (Dai and Mumper, 2010). The present study is an attempt to understand the effects and mechanisms of kaempferol in ALI.

Materials and Methods

Animals and Treatments

All animal experimental protocols were reviewed and approved by the Ethics Committee of Northwest A&F University for the use of Laboratory Animals. Kun-Ming (KM) mice (4 weeks old, 20 ± 2 g of body weight) were purchased from the Fourth Military Medical University (Xi'an, China). All mice were maintained in an air- and humidity-controlled room with a 12 h light and 12 h dark cycle, and the animals had free access to feed and water throughout the experiment.

A total of 30 female mice were randomized into 5 groups of 6 animals each ($n = 6$). (1) Normal control (NC) group received 0.5% carboxymethyl cellulose (CMC) twice per day at a volume of 20 mL/kg of body weight. (2) Alcoholic liver injury (ALI) model group was administrated alcohol by stepwise establishment (Chen *et al.*, 2013). The mice were intragastrically administrated 2, 4, 6, 8 g/kg of distillate spirits (50% alcohol, v/v; Beijing Erguotou Wine Joint Stock Limited Company, China), twice daily at 3 days interval, and finally 10 g/kg body weight for 28 days. The alcohol-intragastric mice had access to their diets at their rations *ad libitum*, and the conditions of different groups were comparable and approximately the same. (3) High concentration kaempferol group (HK) was treated with kaempferol in 0.5% CMC at 20.0 mg/kg of body weight for 28 days twice a day. (4) Low concentration kaempferol-ALI group (LK-ALI) was treated with kaempferol in 0.5% CMC at 10.0 mg/kg and alcohol at 10 g/kg twice daily for 28 days. (5) High concentration Kaempferol-ALI group (HK-ALI) was treated with kaempferol in 0.5% CMC at 20.0 mg/kg and alcohol at 10 g/kg twice daily for 28 days.

Note: alcohol dose $(\mathrm{g}) =$ distillate spirit volume fraction $\times$ liquor volume $(\mathrm{mL}) \times$ relative density of alcohol ($0.93\ \mathrm{g\ mL^{-1}}$).

Collection of Blood Sample and Liver Tissue

Blood samples were collected from heart. The serum was collected by centrifugation at 2500 rpm for 10 minutes at 4°C and stored at −80°C for further analysis. Subsequently, the animals were euthanized by exposure to 10% chloral hydrate and livers were extracted before exsanguination. Immediately after collection, the liver was divided into three parts: 0.5 g for microsomal protein analysis and 0.1 g for mRNA extraction (both samples were stored at −80°C), and a small part was fixed in a buffer solution containing 10% formalin for histopathological analysis.

Biochemical Assays

In order to assess the liver damage, the serum aspartate aminotransferase activity (AST/GOT) and alanine aminotransferase activity (ALT/GPT) were measured at 37°C using AST/GOT and ALT/GPT determination kits (Jiancheng, Inc., Nanjing, China) according to manufacturers' instructions.

Oxidative Stress Assays and Lipid Accumulation

The levels of ROS and anti-oxidative enzymes in serum were determined. The serum Glutathione (GSH) content was assessed by colorization of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reactive GSH at room temperature using GSH determination kits (Jiancheng, Inc., Nanjing, China) and GSH values were expressed as micromoles per liter of serum (Smith *et al.*, 1988). Activity of superoxide dismutase (SOD) and oxidative stress products hydrogen peroxide (H_2O_2) were determined by using commercial kits (Jiancheng, Inc., Nanjing, China). Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels. Serum MDA contents were determined by detecting thiobarbituric acid (TBA) reactive MDA (which is an end product of the peroxidation of polyunsaturated fatty acids and related esters) and the values were expressed as nanomole per milliliter of serum. Quantitative assay of lipids was conducted by measuring the concentrations of serum triglycerides (TGs) using TG content assay kits (Jiancheng, Inc., Nanjing, China).

Histological Examination

The formalin fixed tissues embedded with paraffin and 4-μm sections were prepared and stained with hematoxylin and eosin. The stained areas were viewed under optical microscope (Olympus, Japan) at 400× magnification. Liver histology was assessed by an experienced pathologist without disclosing treatments and groupings information. The pathological changes associated with liver injury were assessed according to the following scale: vacuolation (micro-vesicular 0–4, macro-vesicular 0–4, and total 0–6); disintegration and hydrops (0–4). Results were compared using non-paired statistical analyses and reported as mean ± SD.

Quantitative Real-Time PCR (RT-PCR)

According to the manufacturers' instructions total RNA was extracted from liver tissue using Trizol reagent (Omega, USA). RNA was eluted in 30 μL RNase-free water and its quality and quantity were assessed with an Experion Automated Electrophoresis Station (BioRad, Munich, Germany) using Experion RNA StdSens Analysis Kit (BioRad, Munich, Germany). The RNA quality indicators ranging between 5 and 10 were used. Total RNA (10 μL) from each group was reverse-transcribed using Revert-Aid Kit (Fermentas, USA) according to the manufacturer's instructions using oligo-dT18 primers in a final volume of 20 μL. After terminating cDNA synthesis, each RT reaction was diluted with nuclease-free water to a final volume of 80 μL and stored at −80°C until further use.

Quantification of CYP2E1 transcript was carried out by RT-PCR using Maxima SYBR Green qPCR Master Mix (Promega, Mannheim, Germany). Primers used for CYP2E1 amplification were forward: CTAAGTATCCTCCGTCCTGAC and reverse: GAAGCGTTTCGTAATCGTTG were designed using the Beacon Designer 7.0 (Premier

Biosoft, International) and derived from *Mus musculus* specific CYP2E1 sequences NM.021282. β-actin transcripts were amplified as an internal calibrator using primers forward: CAGCTCTTGGGTACTTCCTT and reverse: TGGAGGTCTTTACGCATAGG (Designed using *Mus musculus* NM.007393). A working primer at a concentration of 10 pmol was used to set a primer matrix experiment to optimize the primer concentrations for valid transcript quantification. RT-PCR reaction mixtures consisted of 10 μL of Cyber green qPCR mix, 2 μL of cDNA template, optimized primer quantities and nuclease free water to make the total reaction volume of 20 μL. Reactions were performed in duplicate for each sample using Light Cycler Software 480 (Roche, Germany). PCR conditions were 95°C for 3 min, then 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 20 s and extension at 72°C for 30 s. No template control reactions were carried to negate PCR contamination and dissociation curve analysis was performed to confirm the authenticity of amplified products. Mean sample threshold cycle values (CT) for CYP2E1 and β-actin were calculated for duplicate samples and relative transcript abundance for target gene expression was calculated using the formula $2^{(-\Delta\Delta CT)}$ (Livak and Schmittgen, 2001).

Western Blot Analysis

Liver microsomal protein ($n = 6$ per group) was harvested from liver tissue and the concentrations of liver microsomal protein were analyzed using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's recommended protocol. Sample proteins were separated using SDS-PAGE with a 12% separating gel by loading equal amounts of protein per lane. The proteins were then transferred to a PVDF membrane under 200 V for 1.5 h. The membrane was blocked with 5% fat-free milk in TBST buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mL Tween 20) for 2 h prior to incubation overnight with anti-CYP2E1 primary antibodies (Boster, China; 1:500) at 4°C. Following the overnight incubations, the membrane was washed three times in TBST buffer and then incubated with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Sungene Biotech, China 1:3000) for 2 h at 37°C. Immunoreactive protein bands were detected using a cECL Western blot kit (Cwbio Inc., China) for the HRP tagged secondary antibody. The bands were scanned and the intensity of protein expression was analyzed using a Gel-pro Analyzer 4.5 (Media Cybernetics, USA).

CYP2E1 Activity Assay

Total liver homogenates from the normal mice were prepared in ice-cold extraction buffer (20 mM Tris-HCl, pH 7.5) using a mechanical homogenizer. Liver microsomal protein concentration was determined using the Lowry method (Lowry *et al.*, 1951). CYP2E1 activity was measured by the rate of oxidation of 1 mM *p*-nitrophenol to *p*-nitrocatechol by 100 g of microsomal protein for 15 min at 37°C (Lu and Cederbaum, 2006). *In vivo*, CYP2E1 enzymes, extracted from different groups, and *p*-nitrophenol

were incubated with NADPH regenerating system in phosphate buffered solution (PBS, 0.05 mol/L, pH 7.4).

CYP2E1 activity was measured both *in vitro* and *in vivo*. Normal mice were euthanized and liver microsomal protein was extracted for the *in vitro* experiment. Part of the extracted CYP2E1 protein was boiled to inactivate enzyme activity and used as negative control. About 14 groups were involved as follows: one negative control group, including inactive CYP2E1 enzyme for triplicate analysis; 13 groups with kaempferol diluted ranging from 2^{-2} to 2^{10} μmol/mL incubated with active CYP2E1 enzyme for triplicate analysis, respectively. CYP2E1 enzyme or inactive CYP2E1 enzyme, *p*-nitrophenol and kaempferol in different doses were incubated with NADPH regenerating system in phosphate buffered solution (PBS, 0.05 mol/L, pH 7.4). The *in vivo* activity of CYP2E1 was measured with the same protocol as described *in vitro*. The inhibition ratio (%) was calculated by the following formula:

$$\text{Inhibition ratio } (\%) = [(\text{A} - \text{B})/\text{A}] \times 100\%$$

where A is the activity of CYP2E1 in the negative control group and B is the activity of CYP2E1 in the kaempferol-treated groups. IC_{50} was calculated by GraphPad Prism 5.0.

Acute Toxicity Study of Kaempferol

Acute toxicity study was performed by oral administration of kaempferol in mice. The mice were randomized into 4 groups, each group consisting of 6 male mice and 6 female mice. The treated mice were administered with kaempferol at doses of 20 mg/kg, 200 mg/kg, and 2,000 mg/kg of body weight for 2 days. The control mice were administered equal volumes of 0.5% CMC. The test solutions were freshly prepared by suspending kaempferol in 0.5% CMC on the day of dosing. The total dose volume was 40 mL/kg daily. Mice were observed for 8 consecutive hours after treatment and then twice a day animals were observed for clinical signs and mortality; this continued for 16 days. Body weight was recorded on the dosing day (D1), at 1 week intervals (D8) and at the end of the study period (D16). Mice were euthanatized as above mentioned for further necropsy.

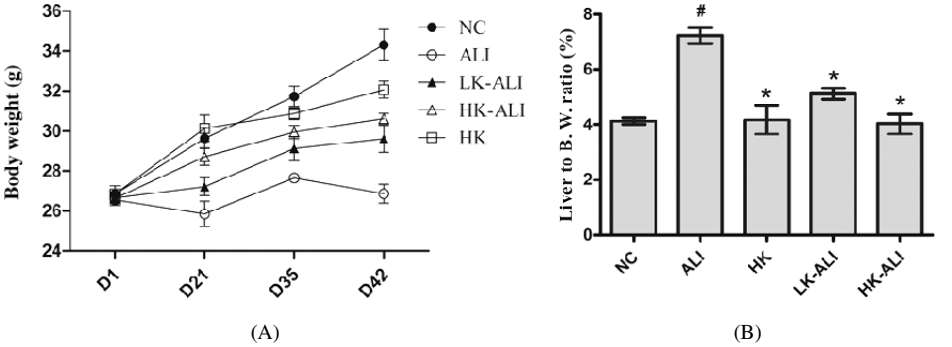
Statistical Analysis

All data were expressed as the mean $\pm$ standard deviation and analyzed using the one-way ANOVA procedure of SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Kaempferol Promoted Body Weight Gain and Liver Ratio Increase

After 4 weeks from the induction of ALI, the average body weight gain in kaempferol-treated groups was significantly higher than in the ALI group (Fig. 1A). The body weight gain ratio in the kaempferol groups was dose-dependent. The high liver ratio induced by alcohol was reduced by kaempferol (Fig. 1B).



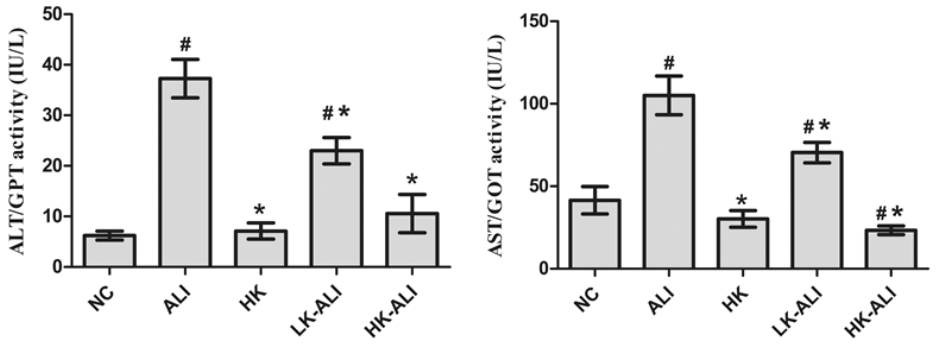
(A) (B)

Figure 1. Body weight development and relative liver ratio. (A) Body weight changes during experimental period; (B) relative liver ratio of different groups. #: significant difference to NC group, $p < 0.05$; * significant difference to ALI group, $p < 0.05$. Groups are abbreviated as follows: NC: normal control; ALI: alcoholic liver injury; LK-ALI: low concentration kaempferol-ALI; HK-ALI: high concentration kaempferol-ALI; HK: high concentration kaempferol. Data are presented as mean ± SD (same as follows).

Kaempferol Attenuated Hepatic Injury

The level of aminotransferases in serum is an indicator for general liver injury. Serum AST/GOT and ALT/GPT were measured in each group with the purpose of correlating the hepatic injury and histological observations. Alcohol significantly induced the levels of both serum AST/GOT and ALT/GPT compared with the control mice. Kaempferol treatment potently alleviated liver injury by reducing the levels of these aminotransferases to their basal levels (Fig. 2). Co-administration of kaempferol reduced these two biochemical markers of ALI in a dose-dependent manner.

The histopathological score was significantly ($p < 0.001$) higher in the ALI group than that of control group (Fig. 3). Both higher and lower doses of kaempferol could improve the liver pathological changes.



(A) (B)

Figure 2. Serum levels of AST/GOT and ALT/GPT. # Denotes significant difference compared to NC group, $p < 0.05$; *denotes significant difference to ALI group, $p < 0.05$.

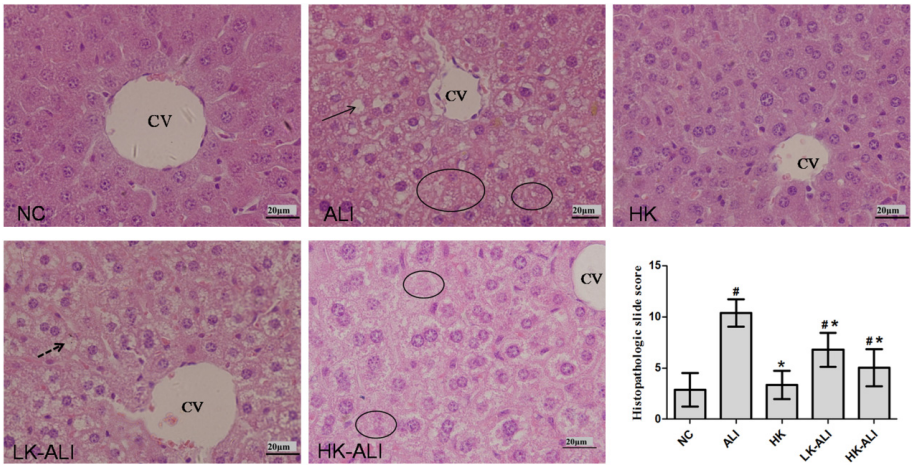
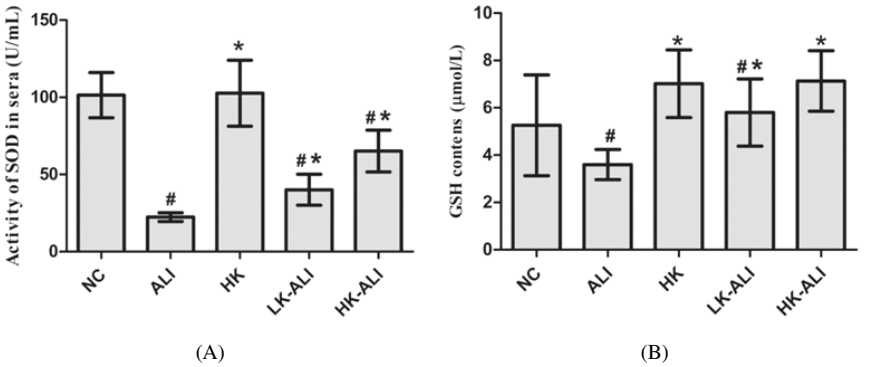


Figure 3. Histopathology of liver sections and scores in mice. Compared to relatively normal morphology in NC group, hepatocellular disintegration, hydrops and vacuolation were observed in ALI group; LK-ALI group showed lighter pathological change; and HK-ALI showed similar morphology to NC group (hematoxylin and eosin stain, original magnification ×400). Histopathologic slide scores were shown as mean ± SD, #denotes significant difference compared to NC group, $p < 0.05$; *: significant difference to ALI group, $p < 0.05$. : Hepatocellular disintegration; : Hydrops; : Vacuolation.

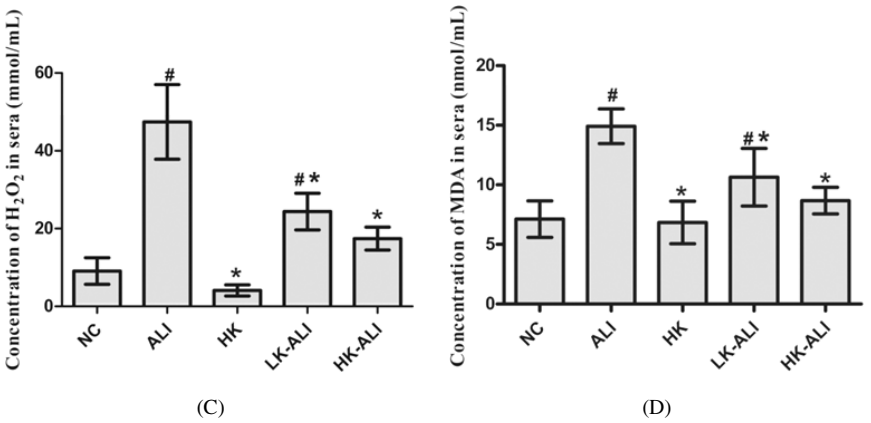
Kaempferol Protected Liver from Oxidative Stress and Lipid Accumulation

The activities of SOD were decreased in the ALI group (Fig. 4A), while they increased in the HK- and LK-ALI groups. The levels of H_2O_2 (Fig. 4C) and MDA (Fig. 4D) were increased in the ALI group with the GSH depletion (Fig. 4B). In the HK- and LK-ALI groups, kaempferol increased the level of GSH and decreased the levels of H_2O_2 and MDA. Meanwhile, serum TG content was reduced by kaempferol treatment.



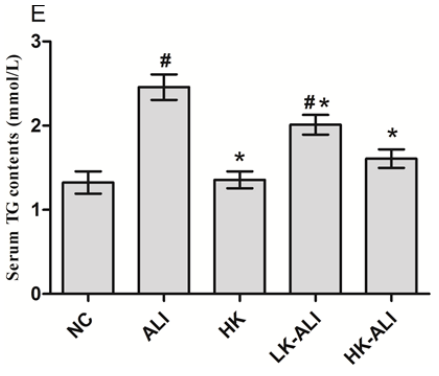
(A) (B)

Figure 4. Effect of kaempferol on oxidative stress and lipid peroxidation. #: Significant difference compared to NC group, $p < 0.05$; *denotes significant difference to ALI group, $p < 0.05$.



(C)

(D)



(E)

Figure 4. (*Continued*)

Kaempferol Inhibited CYP2E1 Expression and Activities

As shown in Figs. 5A and 5B, the mRNA expression and protein levels of CYP2E1 in the ALI group were increased compared with the NC group ($p < 0.005$). In co-treatment with kaempferol for 28 days, the CYP2E1 level was decreased in the HK and HK-ALI groups ($p < 0.005$).

CYP2E1 activity was decreased when it was co-incubated with kaempferol. The inhibition ratio of CYP2E1 was correlated with the dose of kaempferol; the results are presented in Fig. 5C. The IC_{50} of kaempferol was 13.23 μM/mL.

CYP2E1 activity was 2.72 times higher in the ALI group than in the NC group ($p < 0.005$) after 2 weeks of treatment (Fig. 5D). Co-treatment with kaempferol (HK-ALI group) resulted in a 61.23% decrease in CYP2E1 activity compared to that of the ALI group ($p < 0.005$). High dose of kaempferol decreased CYP2E1 activity by 61% compared to that of ALI group ($p < 0.005$; Fig. 5D). No significant differences were observed between the NC and LK groups, and between the HK and ALI groups.

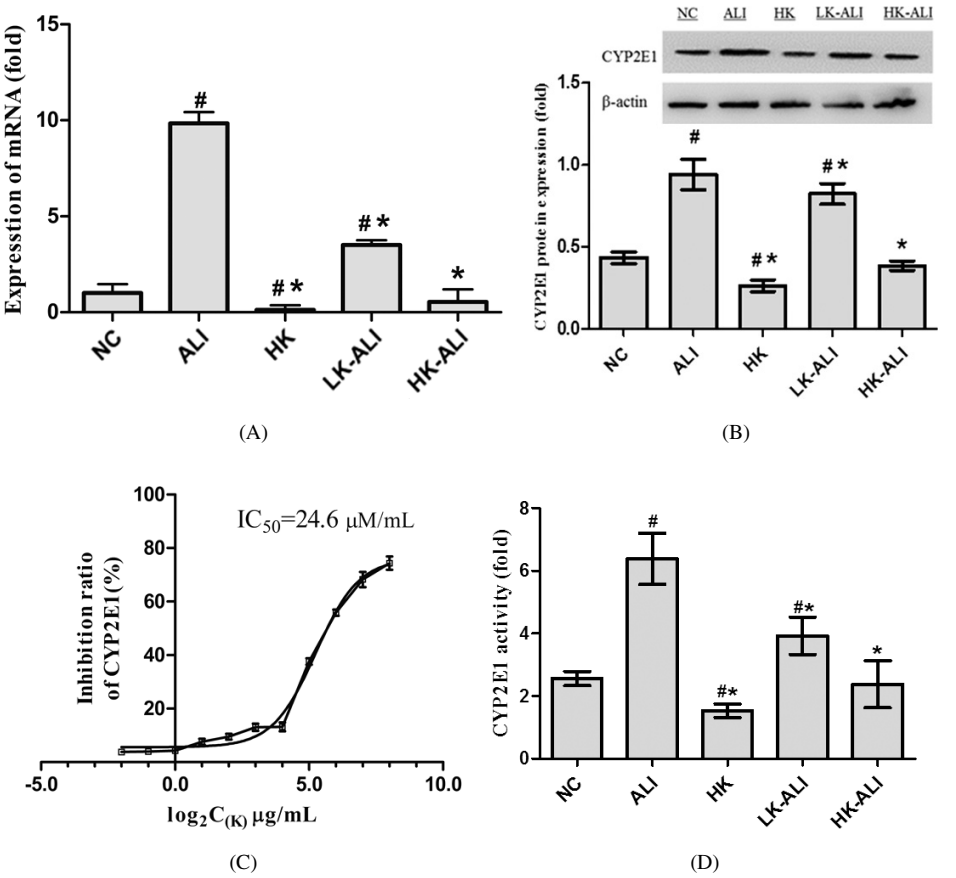


Figure 5. Expression pattern and activity of CYP2E1. Relative mRNA abundance values were measured in different groups and presented in comparison to NC group. CYP2E1 activity was measured by the rate of oxidation of 1 mM *p*-nitrophenol to *p*-nitrocatechol by 100 g of microsomal protein. #: significant difference compared to NC group, $p < 0.05$; *: significant difference to ALI group, $p < 0.05$.

Acute Toxicity Study of Kaempferol in Mice

All mice treated with kaempferol (20 mg/kg, 200 mg/kg, and 2,000 mg/kg) survived the whole study period, and no clinical symptoms were observed during a 14 day acute oral toxicity study. Hypoactivity and piloerection were observed in female mice within 3.5 h after administration of 2,000 mg/kg kaempferol. Discolored semi-solid stool was observed 3.5–4.5 h after administration of 2,000 mg/kg kaempferol. All clinical signs recovered on the second day. No statistically significant differences in body weights were observed between the treatment and control groups during the study period. No gross lesions were detected in any animal upon necropsy.

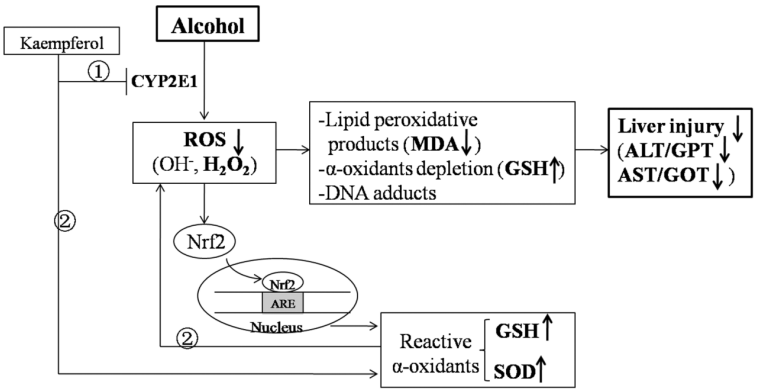


Figure 6. Summary of kaempferol effect in ALI intervention. The mechanism of kaempferol in protecting ALI probably is related to two aspects: ① the ROS generation is reduced by inhibiting CYP2E1; ② the ROS is removed by enhancing anti-oxidant defense. Co-administration of kaempferol down-regulates CYP2E1 with the ROS generation reducing. Reactive anti-oxidants enzymes (GSH and SOD) were induced to the clearance of lipid products (MDA) and ROS (H_2O_2). Liver function (ALT/GPT and AST/GOT) is improved by reducing the oxidative stress. All the arrows represent various indices changes in ALI model mice treated with kaempferol.

Discussion

In the current study, alcohol treatment significantly induced the level of ALT/GPT and AST/GOT in serum, this observation indicated that the ALI model had been successfully constructed in mice (Kasdallah-Grissa *et al.*, 2007). The fact that kaempferol treatment noticeably decreased serum ALT/GPT and AST/GOT demonstrated kaempferol's protective effect against ALI.

The elimination of alcohol is primarily dependent on the oxidation of alcohol ADH and CYP2E1 (Lieber, 2004). High doses of alcohol are primarily oxidized by CYP2E1, a major oxidative stress contributor to the production of additional toxic ROS (Leung and Nieto, 2013). Oxidative stress and lipid peroxidation by CYP2E1 induction is known to be a major consequence of ethanol-mediated liver toxicity. Recent studies have indicated that kaempferol has liver protective effects against oxidative stress (Tatsimo *et al.*, 2012) and lipid accumulation (Nirmala and Ramanathan, 2011). A decrease of CYP2E1 induction was associated with a reduction in alcohol-induced liver injury in mice (Lu *et al.*, 2008). In the present study, female mice were used because they are highly sensitive to CYP2E1 up-regulation compared to male mice (Lu *et al.*, 2011). As presented in Fig. 5, kaempferol was able to inhibit CYP2E1 in both expression level and activity, therefore leading to a decreased generation of ROS (H_2O_2 and MDA). These findings were consistent with the kaempferol-induced suppression of CYP2E1 observed in other liver damage disease models (Shih *et al.*, 2013), as CYP2E1 is also involved in the metabolism of other toxins in addition to alcohol. Alcohol-induced CYP2E1 is triggered by specificity protein 1 (SP1), a transcription factor which binds to the promoter of CYP2E1 (Peng and Coon, 2000). It has been demonstrated that mithramycin A, an inhibitor of SP1, can completely abrogate the induction of CYP2E1 by ethanol in SVGA astrocytes (Jin *et al.*, 2013). Therefore, it is

important to further reveal the possible effect of kaempferol on SP1 in terms of the CYP2E1 inhibition.

The activation of the ROS-removing enzymes (SOD and GSH) also plays a role in the reduction of ROS (Liao *et al.*, 2013; Na *et al.*, 2013). The concentrations of GSH and the activity of SOD in the LK-ALI and HK-ALI groups were significantly increased, resulting in decrease of the oxidative products, including MDA and H_2O_2. These findings were in agreement with those of previous studies (Jung *et al.*, 2009; Bhouri *et al.*, 2011). Furthermore, alcohol accelerated lipid accumulation in the liver, while kaempferol could lessen the fatty liver injury as shown by histopathology and serum TG test. Hence, it indicated that kaempferol exerts an inhibitory effect on lipid oxidation and attenuates oxidative stress in the ALI (Fig. 6).

Conjugated kaempferol and two major metabolites (quercetin and isorhamnetin) and their glucuronides were found in the systemic plasma and the portal plasma after oral administration of 100 mg/kg kaempferol, although their bioactivities remained unclear (Barve *et al.*, 2009). Although it is reported to have low oral bioavailability (2–20%), our study proved that oral kaempferol dosed 10–20 mg/kg can effectively reduce the risk of ALI and there was no significant difference compared to the kaempferol treatment by intraperitoneal injection. Kaempferol has prominent phenolic OH groups, which could be the structural basis of ROS scavenging (Priyadarsini *et al.*, 2003).

Natural products with hepatoprotective activity have been applied to protect various liver disorders (Gou *et al.*, 2013; Li *et al.*, 2013), however only few of them have been proven for hepatoprotective activities against ALI (Adewusi and Afolayan, 2010). Silymarin is the most popular drug against ALI despite of a controversy about its effect. Some Cochrane systematic reviews and meta-analysis studies concluded that silymarin does not significantly influence the course of patients with ALI (Rambaldi *et al.*, 2005). Antioxidants were also applied as complementary strategies in ALI therapy. The effects of *N*-acetyl-cysteine (NAC) and vitamin C (Vc) on ROS clearance have been demonstrated by the fact that hepatic GSH was increased in mice treated with NAC and Vc, respectively (Lu *et al.*, 2012). However, in our study, kaempferol could not only increase the antioxidative defense, but also reduce the ROS generation by inhibiting CYP2E1. According to a recent study, kaempferol could lessen the lipid accumulation in non-alcoholic fatty liver disease (NAFLD), but there is no such data in ALI treatment yet (Wei *et al.*, 2014). It is suggested that kaempferol could be a promising natural compound in ALI intervention and this may broaden our approach in searching ALD relevant natural products.

Acknowledgments

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