Original Article

Escin protects against acetaminophen-induced liver injury in mice via attenuating inflammatory response and inhibiting ERK signaling pathway

Hung-Chen Lee1,2,3*, Huang-Ping Yu1,2*, Chia-Chih Liao1,2, An-Hsun Chou1,2, Fu-Chao Liu1,2

1Department of Anesthesiology, Chang Gung Memorial Hospital, Taoyuan, Taiwan; 2College of Medicine, Chang Gung University, Taoyuan, Taiwan; 3Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taoyuan, Taiwan. *Equal contributors and co-first authors.

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Abstract: Acetaminophen (APAP) overdose may lead to the formation of oxidative stress, hepatocyte apoptosis and necrosis, and, eventually result in acute liver failure. Escin, a major extracted component of Aesculus hippocastanum, reportedly exerts anti-inflammatory, anti-edematous and anti-oxidant properties. Previous studies have demonstrated these protective effects of A. hippocastanum extracts on ischemia/reperfusion intestinal injury and endotoxin-induced lung injury. In this study, we aimed to evaluate the effect of escin on APAP-induced liver injury in mice. Mice were intraperitoneally administrated with APAP (300 mg/kg) or an equal volume of saline (control), followed by a treatment with various concentrations of escin (0, 0.5, 1, 2 and 4 mg/kg) for 30 min. The animals were sacrificed 16 h following APAP administration for serum and liver tissue assay. Escin treatment attenuated the damage of APAP-induced liver injury in a dose-dependent manner (0.5-4 mg/kg). Escin also attenuated the hepatic myeloperoxidase (MPO) activity and hepatic pro-inflammatory cytokines (i.e., TNF-α, IL-1β, IL-6 and IL-17). Furthermore, escin treatment decreased the hepatic phosphorylation expression of extracellular signal-regulated kinase (ERK). Our data indicates that escin shows protective effects on APAP-induced hepatotoxicity in a dose-dependent manner through anti-inflammatory mechanism and the inhibition of ERK signaling pathway.

Keywords: Acetaminophen, liver injury, escin, inflammation, pro-inflammatory cytokine, ERK

Introduction

Acetaminophen (N-acetyl-p-aminophenol; APAP) is very commonly used for its antipyretic and analgesic properties across several countries. Although APAP is generally considered safe, its overdose is the leading cause of drug-induced hepatotoxicity and fulminant hepatic failure in numerous countries [1]. At recommended doses, APAP can be primarily metabolized by glucuronidation and sulfuration in hepatocytes for forming non-toxic metabolites, while a small amount of APAP can be subsequently bioactivated by cytochrome P450 enzymes to form a toxic byproduct, N-acetyl-p-benzoquinone imine (NAPQI), which is rapidly conjugated with glutathione (GSH) [2]. However, in the case of APAP overdose, excessive NAPQI results in GSH depletion and covalent binding to cellular macromolecules, subsequent mitochondrial dysfunction, oxidative stress and DNA fragmentation, which eventually leads to hepatocyte apoptosis and necrosis [3, 4].

Additionally, it is well-established that the innate immune immunity plays a key role in the progression and amplification of APAP-induced hepatotoxicity [5, 6]. Following the initial hepatocytes necrosis and parenchymal damage, damage-associated molecular patterns (DAMPs) are recognized by resident liver macrophages (Kupffer cells, KC) [7]. Furthermore, active KC produces pro-inflammatory and chemotactic cytokines, which results in the infiltration and activation of neutrophils and macrophages into the damaged liver tissues, which leads to aggravated liver injury [6, 8].

Moreover, numerous studies have revealed that the mitogen-activated protein kinase (MAPK) signaling pathway, which regulates the intracellular signal transduction to extracellular signals,
is essential for APAP-induced liver injury [9, 10]. Studies have also demonstrated that the extracellular signal-regulated kinase (ERK) signaling pathway, one of the MAPK family, participates in the mitochondrial dysfunction, oxidative stress, cell apoptosis and inflammation [11, 12]. Moreover, the inhibition of ERK signaling pathway attenuates the inflammatory process during APAP-induced liver injury [9, 13].

Escin—a major extracted component of Aesculus hippocastanum—reportedly displays anti-inflammatory, anti-edematous and anti-oxidant properties [14, 15]. Accordingly, escin has been widely used in the clinical treatment of hemorrhoids, traumatic edema and chronic venous insufficiency [16]. Previous studies have demonstrated that escin imparts protective effects on endotoxin-induced liver and lung injury by downregulating the levels of pro-inflammatory cytokines, inhibiting the inflammatory response and improving the survival rate [17, 18]. Moreover, recent studies have found that a number of natural products obtained from medicinal plants show protective effects against multiple organ dysfunctions [19], acute lung injury model [20], as well as liver injury model [21, 22]. However, whether escin possesses protective effects against APAP-induced liver injury remains unclear. Therefore, we investigated the effect of escin on APAP-induced hepatotoxicity in mice by assessing the degree of liver injury, neutrophil and macrophage infiltration, myeloperoxidase (MPO) expression, pro-inflammatory cytokine levels and the upregulation of MAPK signaling pathway.

**Materials and methods**

**Animals**

Adult male C57BL/6C (B6) mice (age, 8-10 weeks; weight, 20-24 g) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). All mice were housed in environmentally controlled facility with a 12 h light/dark cycle and allowed ad libitum access to sterile food and clean water. All animal experimental procedures were performed in accordance with the guidelines of the Animal Welfare Act and Guide for Care and Use of Laboratory Animals from the National Institute of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Chang Gung Memorial Hospital.

**Experimental model and treatments**

The experimental mice were intraperitoneally injected with 300 mg/kg APAP [23, 24] (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in warm saline (20 m/mL), while the control group received intraperitoneal (i.p.) equal volume of normal saline. After 30 min of injection, the mice were treated with i.p. equal volume of phosphate-buffered saline (PBS) or escin (Sigma-Aldrich Co., St. Louis, MO, USA) at a concentration of 0.5, 1, 2 or 4 mg/kg [25, 26]. All mice were sacrificed following 16 h of APAP or normal saline i.p. injection under anesthesia by i.p. administered 100 mg/kg body weight ketamine and 10 mg/kg body weight xylazine for the analysis of blood sample and liver tissues.

**Measurement of alanine transaminase level in the serum**

Blood samples were collected through cardiac puncture and left to stand for 1 h at room temperature, followed by centrifugation at 12000 g for 5 min twice. The serum alanine transaminase (ALT) levels were measured using the VITROS DT60 II Chemistry System (Ortho-Clinical Diagnostics, Raritan, NJ, USA).

**Hepatic myeloperoxidase assay**

Myeloperoxidase (MPO) acts a biomarker of oxidative stress and inflammation in inflammatory process. Briefly, the liver tissues were homogenized in buffer (50 mM potassium phosphate [KPO₄] buffer [pH 7.4]) on ice. Next, the tissue homogenate was centrifuged at 15000 g for 15 min at 4°C, followed by washing twice and being resuspended in buffer (50 mM KPO₄ buffer [pH 6.0]) at room temperature, and then incubated for 2 h at 60°C. Lastly, the homogenate was sonicated for 10 s and underwent 3 cycles of freeze/thaw. Samples were centrifuged at 15000 g for 15 min at 4°C, and the supernatant was added to 50 mM KPO₄ (pH 6.0) buffer containing o-dianisidine (10 mg/mL), 0.3% hydrogen peroxide (H₂O₂). The absorbance at 460 nm of the final solution was measured over 5 min.

**Histology and immunohistochemistry**

The left lobes of the liver were excised and fixed in 4% paraformaldehyde and embedded in paraffin. Sections (of 4-μm thickness) were stained using hematoxylin and eosin (H&E) for histolo-
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For evaluating the immune reaction at the area of inflammation, we measured 4 pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-17. The liver tissues were homogenized in lysis buffer (20 mM HEPES, 1 mM 2-ME, 3 mM MgCl$_2$, 150 mM NaCl, 1 M DTT, 0.1 mM PMSF, 0.25 µg leupeptin, 0.05 µg pepstatin A and 0.01 µg aprotinin). The homogenized tissues were sonicated for 15 s, and then centrifuged at 12000 g for 10 min at 4°C. 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for separating the protein, followed by electroblotting onto polyvinylidene fluoride (PVDF) membrane. After blocking in 5% fat-free milk solution for 1 h and rinsing thrice with Tris-buffer (1% Tween-20), the membrane was incubated with specific primary antibody against ERK, c-Jun N-terminal kinase (JNK), p38, phospho-ERK, phospho-JNK and phospho-p38 (1:1000; Cell Signaling Technology, MA, USA) for 1 h at room temperature, the immune complexes were visualized using the enhanced chemiluminescence (ECL) system. The antibody against β-actin (Proteintech Group, Inc., Chicago, IL, USA) were used for confirming the loading accuracy.

Statistical analysis

All data were presented as mean ± standard error of mean (SEM) (n=5-6 mice for each group). One-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests were used to analyze the results. The Prism 6.0 Software (GraphPad Software Inc., San Diego, CA, USA) was used for performing the statistical analyses. P<0.05 was considered to indicate statistical significance.

Results

Effects of escin on ALT levels in the serum after APAP-induced liver injury

The serum ALT level was markedly increased in the APAP (300 mg/kg) group than in the control group (P<0.01). No difference was noted in the serum ALT levels between the control and escin alone groups. Furthermore, after 30 min of APAP administration, escin (2 and 4 mg/kg, P<0.01) treatment group showed significantly attenuated serum ALT levels than the APAP group (Figure 1). However, when treated with lower dose of escin (0.5 and 1 mg/kg), the serum ALT levels did not decrease significantly.
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Figure 2. Effects of escin on histological changes in APAP-induced liver injury. Mice were intraperitoneally administered with (A) control (normal saline), (B) escin (4 mg/kg) alone, (C) APAP (300 mg/kg) alone or treated with various concentrations of escin [(D) 0.5 mg/kg, (E) 1 mg/kg, (F) 2 mg/kg, and (G) 4 mg/kg] after 30 min of APAP administration. Representative histological changes of liver obtained from different groups. (50× and 100× magnifications are shown).

This data suggests that escin treatment can protect against APAP-induced hepatotoxicity in a dose-dependent manner.

Effects of escin on histological changes in the liver

Histological examination, which confirmed the APAP-induced liver injuries, demonstrated severe centrilobular necrosis, sinusoidal congestion and fatty infiltration in the APAP group. The histological appearances of liver tissues in the control and escin alone groups appeared normal. Moreover, treatment with escin (2 and 4 mg/kg) evidently attenuated these pathological findings following APAP-induced hepatotoxicity (Figure 2). These findings indicated that escin treatment could ameliorate APAP-induced liver injury.

Effects of escin on MPO activity in liver tissues

MPO activity, a biomarker to indicate the degree of neutrophil accumulation and inflammatory response, was obviously elevated in the APAP (300 mg/kg) group than in the control group (P<0.01). No difference was noted in the hepatic MPO activity between the control and escin alone groups. Moreover, after 30 min of APAP administration, escin (2 and 4 mg/kg, P<0.01) treated group showed significantly decreased hepatic MPO activity than the APAP group, but no effect was observed when treated with a lower dose of escin (0.5 and 1 mg/kg) (Figure 3). This result demonstrates that escin treatment dose-dependently reduces neutrophil accumulation in the liver.
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Effects of escin on the infiltration of neutrophil in the liver

Immunohistochemical staining with Ly6G antibody, a specific marker for granulocyte, was used to investigate the inflammatory infiltration of neutrophils in APAP-induced liver injury. APAP-treated animals demonstrated dominant infiltrated neutrophils in the hepatic necrotic area as compared with the control animals. The escin (2 and 4 mg/kg) treated group showed significantly reduced hepatic neutrophil accumulation in the liver parenchyma after 30 min of APAP injection than the APAP group did (Figure 4).

Effects of escin on infiltration of macrophages in the liver

Immunohistochemical staining with Mac-2 antibody, a specific marker for macrophage, was used to investigate infiltration and accumulation of macrophages in APAP-induced liver injury. APAP-treated animals demonstrated obviously infiltrated macrophages in the hepatic injured area than the control animals did. Moreover, escin (2 and 4 mg/kg)-treated group showed significantly decreased hepatic macrophage infiltration in the liver parenchyma after 30 min of APAP injection than the APAP group did (Figure 5).

Effects of escin on hepatic TNF-α, IL-1β, IL-6 and IL-17 levels

For evaluating the effects of escin on pro-inflammatory cytokine expression in APAP-induced hepatotoxicity, we measured the TNF-α, IL-1β, IL-6 and IL-17 levels in the liver using ELISA. The hepatic levels of TNF-α, IL-1β, IL-6 and IL-17 were significantly elevated in the APAP group than in the control group. No difference was observed in these pro-inflammatory cytokine levels between the control and escin alone groups. Moreover, after 30 min of APAP challenge, treatment with escin (2 mg/kg) significantly decreased the hepatic TNF-α and IL-6 levels (P<0.05) than treatment with APAP. Furthermore, treatment with a higher dose of escin (4 mg/kg) significantly attenuated hepatic TNF-α, IL-1β, IL-6 and IL-17 levels (P<0.05, P<0.05, P<0.01, P<0.05, respectively) than treatment with APAP (Figure 6). These datum indicated that escin treatment reduced the production and release of pro-inflammatory cytokines in APAP-induced hepatotoxicity in a dose-dependent manner.

Effects of escin on ERK, JNK and p38 expression and phosphorylation in the liver

We further investigated the hepatic MAPK family protein expression and its phosphorylation in APAP-induced liver injury. No significant difference was observed in the hepatic ERK, JNK and p38 protein expressions between the control and escin alone groups. The ERK activity, as determined by its phosphorylation, was markedly increased in the APAP group than in the control group. Moreover, escin (1, 2 and 4 mg/kg) treatment after 30 min of APAP challenge evidently decreased the phosphorylated ERK expression than APAP treatment (Figure 7). However, no significant difference was observed in the hepatic JNK and p38 protein expressions and phosphorylation among these 7 groups. Cumulatively, our results revealed that escin treatment dose-dependently attenuated the ERK phosphorylation and activation in the liver during APAP-induced hepatotoxicity.

Discussion

In the present study, we investigated the protective effects of escin, a major active component extracted from A. hippocastanum, on APAP-induced liver injury in mice. After APAP
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Figure 4. Effects of escin on hepatic neutrophil infiltration in APAP-induced liver injury. Mice were intraperitoneally administered with (A) control (normal saline), (B) escin (4 mg/kg) alone, (C) APAP (300 mg/kg) alone or treated with various concentrations of escin [(D) 0.5 mg/kg, (E) 1 mg/kg, (F) 2 mg/kg, and (G) 4 mg/kg] after 30 min of APAP administration. The liver sections were immunostained with Ly6G antibody (brown). Representative immunohistochemical staining of liver obtained from different groups. (100× and 200× magnifications are shown). Quantification of Ly6G-positive cells was analyzed under high power field (HPF). Each value represents mean ± SEM; n=5-6 for each group. ***P<0.005 vs. control group; **P<0.01, ***P<0.005 vs. APAP group.
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(300 mg/kg) challenge for 16 h for generating hepatotoxicity, the ALT levels, hepatic MPO expression, immune cell infiltration, as well as pro-inflammatory cytokine expression were fo-
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To markedly increase. Treatment with escin after 30 min of APAP challenge evidently decreased these inflammatory parameters. Briefly, escin treatment dose-dependently attenuated the damage to APAP-induced liver injury, decreased neutrophil and macrophage infiltrations, and also declined the hepatic pro-inflammatory cytokines (i.e., TNF-α, IL-1β, IL-6 and IL-17) levels. Moreover, escin (1-4 mg/kg) treatment obviously decreased the phosphorylation of the hepatic ERK protein. Taken together, our results suggest that escin induced protective effects against APAP-induced hepatotoxicity in mice in a dose-dependent manner as a consequence of anti-inflammatory mechanism and the inhibition of ERK signaling pathway.

There is a growing evidence that innate immune plays a crucial role in the amplification and aggravation of APAP-induced liver injury [5, 6]. Hepatocyte death during APAP-induced hepatotoxicity results in the release of DAMPs while hepatocytes undergo apoptosis or necrosis [7]. DAMPs are recognized by the resident KC as well as infiltrating macrophages through toll-like receptors [27], and later, these cells become activated [7, 28]. Subsequently, activated macrophages secrete C-X-C motif (CXC) chemokines, which facilitate the recruitment of monocytes and neutrophils into the liver [8, 29]. Several studies have indicated that APAP-induced liver injury is characterized by extensive inflammatory responses and pro-inflam-
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Previous studies also reported that escin could downregulate the inflammatory gene expression and decrease the pro-inflammatory cytokines (i.e., TNF-α, IL-1β and IL-6) levels in an ischemic or lipopolysaccharide (LPS) model [15, 31]. Moreover, a recent study revealed that escin inhibited high-mobility group box 1 (HMGB1) release from macrophages and increased the survival rate in LPS-induced mice [25]. In the current study, escin treatment attenuated APAP-induced liver injury, accompanied by a decrease in the level of hepatic pro-inflammatory cytokines (i.e., TNF-α, IL-1β, IL-6 and IL-17) in compared with APAP treatment. The possible protective effects of escin on APAP-induced hepatotoxicity maybe mediated by reduced activation and accumulation of macrophages (less DAMPs stimulus), resulting in the suppression of the production of pro-inflammatory cytokines and, thereby, attenuating neutrophil infiltration and activation.

Figure 7. Effects of escin on the hepatic ERK (A), JNK (B), p38 (C) expression and phosphorylation in APAP-induced liver injury. Mice were intraperitoneally administrated with APAP (300 mg/kg) or an equal volume of saline (control), and treated with various concentrations of escin (0, 0.5, 1, 2 and 4 mg/kg) for 30 min. Equal protein loading is illustrated by the β-actin bands. The bands were analyzed using densitometry. Each value represents mean ± SEM; n=5-6 for each group. **P<0.01, ***P<0.005 vs. control group; ***P<0.005 vs. APAP group.
It is well-established that IL-17 is a major pro-inflammatory cytokine playing a pivotal role in recruiting the immune cell, especially neutrophils, to the inflammatory area [32, 33]. Several innate immune cells, such as nature killer cells, NKT cells as well as γδ T cells, could produce and release IL-17 [34]. Previous studies also demonstrated that IL-17 facilitate neutrophil recruitment and accumulation by upregulating the chemokines expression in peritoneal inflammation, sepsis model [35], and ulcerative colitis patients [36]. In addition, the deficiency of IL-17 protected from the kidney [33] and liver [37] ischemia-reperfusion injury, accompanied by the attenuation of the degree of neutrophils infiltration and the release of pro-inflammatory cytokines. A recent study showed that IL-17-producing γδ T cells mainly contribute to neutrophil activation and infiltration and the subsequent inflammatory response in a APAP-induced liver injury model [38]. Moreover, the levels of IL-17 were obviously elevated after diclofenac overdose in a diclofenac-induced liver injury model, while treatment with IL-17 monoclonal antibody resulted in reduced hepatic damage and less pro-inflammatory cytokine production [39]. Recently, IL-17 knockout mice were used in an APAP-induced liver injury model and demonstrated that IL-17 deficiency significantly reduced APAP-induced hepatotoxicity associated with decreased immune response, neutrophil recruitment and pro-inflammatory cytokines expression [24]. Our current study found that the level of hepatic IL-17 was significantly elevated after APAP challenge, while escin treatment could reduce the level of hepatic IL-17. These datum collectively suggested that escin treatment decreased the expression of IL-17 and the subsequent less degree of neutrophil activation and infiltration, which resulted in attenuated inflammatory response as well as hepatocyte death.

The MAPK family proteins contribute to the regulation of diverse cellular programs by transmitting extracellular stimulus to intracellular responses. ERK is one of the MAPK pathways and associated with mitochondrial malfunction, oxidative stress, apoptosis [11], as well as immune response [12]. Previous studies also found that the MAPK pathway was relevant to APAP-induced hepatotoxicity [9, 10]. The administration with ERK inhibitor protects against APAP-induced hepatotoxicity in mice accompanied by decreased reactive oxygen species (ROS) formation [9, 13]. Recent studies also demonstrated that stimuli due to ROS and DNA damage could upregulate the ERK signaling pathway [40, 41]. Moreover, TNF-α can activate MAPK, thereby resulting in apoptosis through JNK and ERK signaling pathways in hepatic injury [42, 43]. It is also well-known that apoptosis can contribute to APAP-induced liver injury [6, 8]. Furthermore, recent studies also demonstrated that ERK-dependent activation of NF-kB can lead to the upregulation of the gene of pro-inflammatory cytokine [44, 45]. In this experiment, treatment with escin after 30 min of APAP challenge dramatically attenuate hepatic ERK phosphorylation, revealing that...
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Escin suppresses the ERK activity and results in less hepatocyte apoptosis and downregulation of pro-inflammatory cytokines in APAP-induced liver injury. Our data suggests that escin treatment protects against APAP-induced liver injury via the inhibition of the ERK signaling pathway.

This study had some potential limitations. First, escin was administered after 30 min of APAP injection. Escin was not given at a later time point after APAP administration. It remains as a potential limitation of the study. Second, we measured serum enzymes and histology; however, we did not measure at survival or any specific hepatic function. It can also be a limitation of the study.

In conclusion, our data indicated that escin had protective effects against APAP-induced hepatotoxicity in a dose-dependent manner through anti-inflammatory mechanism such as attenuated inflammatory immune cell infiltration and decreased pro-inflammatory cytokine production, as well as the inhibition of ERK signaling pathway (Figure 8). Cumulatively, the present study suggests that escin may be a potential drug target for APAP-induced liver injury. Further focused studies are required for confirming its clinical applications and side effects.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Fu-Chao Liu, Department of Anesthesiology, Chang Gung Memorial Hospital and Chang Gung College of Medicine, No. 5, Fuxing St., Guishan Dist., Taoyuan 333, Taiwan. Tel: +886-3-3281200 Ext. 3624; Fax: +886-3-3281200 Ext. 2787; E-mail: ana5189@cgmh.org.tw

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