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Protective effects of luteolin against acetaminophen-induced acute liver failure in mouse

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ABSTRACT

Acetaminophen (APAP) is widely used as a safety analgesic and antipyretic agent. Although considered safe at therapeutic doses, overdose of APAP can cause acute liver injury that is sometimes fatal, requiring efficient pharmacological intervention. Luteolin is a naturally occurring flavonoid which is abundant in plants. The objective of this study was to investigate corresponding anti-oxidative and anti-inflammatory activities of luteolin, using acetaminophen-treated mice as a model system. Male C57BL/C mice were randomly divided into three groups (n = 6 each). The control group was given phosphate buffered saline (PBS) orally. The APAP group was given APAP by intraperitoneal injection (i.p) at 300 mg/kg suspended in PBS. The luteolin-treated group was given APAP and luteolin (0–100 mg/kg/day, 1 or 3 days before APAP administration) suspended in PBS orally. 16 h after APAP administration, the liver and serum were collected to determine the liver injury. Luteolin administration significantly decreased acetaminophen-induced serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), malondialdehyde (MDA) levels, as well as glutathione (GSH) depletion and decrease of superoxide dismutase (SOD). Luteolin restored SOD, GSH and GSH-px activities and depressed the expression of pro-inflammatory factors, such as inducible nitric oxide synthase (i-NOS), TNF-α, nuclear factor kappa B (NF-κB), and IL-6, respectively. Moreover, luteolin down-regulated acetaminophen-induced nitrotyrosine (NT) formation and endoplasmic reticulum (ER) stress. These results suggest the presence of anti-oxidative, anti-inflammatory and anti-ER stress properties of luteolin in response to acetaminophen-induced liver injury in mice.

1. Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug, and overdoses of it can cause hepatic necrosis and renal failure. APAP poisoning accounts for more than 30,000 hospital admissions and approximately 500 deaths every year in the USA [1]. APAP-induced acute liver failure (ALF) is a sterile inflammatory condition, with local and systemic inflammatory responses mediated by the release of pro-inflammatory cytokines from innate immune cells and activation and migration of macrophages into the liver [2,3]. APAP is metabolized by cytochrome P450 to produce N-acetyl-p-benzoquinoneimine (NAPQI), which can react with glutathione (GSH) to cause oxidative stress that may trigger the mitochondrial signal pathway and lead to cell injury [4]. Mitochondrial damage is a well-known effect caused by APAP, which can inhibit mitochondrial respiration and decrease membrane potential to produce mitochondrial dysfunction and oxidant stress. In addition, some biological processes including apoptosis, inflammatory and autophagy have been found in APAP-induced hepatotoxicity [5,6].

Luteolin (3, 4, 5, 7-tetrahydroxyflavone) is a naturally occurring flavonoid, abundant in plants worldwide such as fruits, vegetables, and certain herbal medicines [7]. Since its isolation, luteolin has shown various biological effects including anti-inflammatory and antioxidant properties, as well as its anti-proliferative activities against various cancer cells [8–11]. Recently, luteolin has also been shown to exhibit a therapeutic effect on liver injury induced by tetrachloromethane (CCl_4), ethanol in mice [12,13].

The main purpose of this study was to investigate the potential effects of luteolin in reducing oxidative stress and inflammation in the liver of mice caused by APAP, as well as enhancement of hepatic proliferative capability, which could provide helpful information for the prevention of liver damage. The present investigation focuses on evaluation of the efficacy of luteolin for its protection against APAP-induced hepatotoxicity.

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2. Materials and methods

2.1. Experimental animals and reagents

The study was conducted using male C57BL/6 mice (4–5 weeks old, 21–26 g) (Animal Feeding Center of Xi'an Jiaotong University Medical School). All mice were housed (5 per cage) in clear, pathogen-free polycarbonate cages in the animal care facility, and were fed a standard animal diet and water ad libitum under controlled temperature conditions with 12-hour light–dark cycles. They were cared in accordance with the Ethical Committee, Xi'an Jiaotong University Medical School. Luteolin was obtained from Dinuo Technologies Co. Ltd (Xi'an, China; ≥98%).

2.2. Study design

In the experiment, acute liver injury (ALI) was induced by a sublethal dose of APAP (300 mg/kg dissolved in 1 mL sterile saline) administered by intraperitoneal (i.p.) injection [14]. Animals were divided into three groups as follows: (1) the normal control group: male C57BL/6 mice remained untreated; (2) the APAP group: male C57BL/6 mice were treated i.p with physiological saline for 3 days before APAP challenge; and (3) the APAP + luteolin group: male C57BL/6 mice were treated i.p with luteolin for 3 days before APAP challenge. Luteolin dissolved in PBS was administered orally at 0–100 mg/kg, and once daily for 1 day or 3 consecutive days. Six mice per group were used in this study. Mice were sacrificed at 16 h after APAP administration and blood samples were collected from the eyeballs. Serum was separated by centrifugation at 4 °C, 3000 ×*g* for 15 min. The liver was removed immediately from each mouse, and kept at −80 °C until analyzed.

2.3. Measurement of liver function

Activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by an automated procedure in the Department of Inspection, The First Affiliated Hospital of Xi'an Jiaotong University [15].

2.4. Cytokine measurement in murine serum

Levels of serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured with commercial ELISA kits following the instructions of the manufacturer (Dakewe, Shenzhen, China).

2.5. Liver enzymatic activity assay

Livers were removed and washed with ice-cold phosphate buffered saline (PBS), blotted and weighed, and then a tissue homogenate (10%, w/v) was prepared in normal saline. The homogenates were then centrifuged at 4000 rpm (4 °C) for 20 min to collect supernatants for determination of GSH, glutathione peroxidase (GSH-px), superoxide dismutase (SOD) and malondialdehyde (MDA) contents. They were measured using the activity assay kits from NanJing JianCheng Bioengineering Institute, using methods as described previously [15].

2.6. Histological study

Samples from the liver were fixed in 10% formalin solution and embedded in paraffin. Serial sections of 5-μm thickness were obtained and stained with hematoxylin/eosin (H&E) to evaluate morphology. The results were examined in a blinded fashion by two researchers.

2.7. Immunohistochemistry (IHC)

The paraffin-embedded sections were deparaffinized and rehydrated and were treated with a 1 mM EDTA buffer (pH = 9.0) in a microwave for 3 min for antigen retrieval. The following steps were performed per the instructions of Histostain TM-Plus and diaminobenzidine substrate kits. Briefly, 3% H_2O_2 was used to block endogenous peroxidase activity. Nonspecific protein binding was blocked by normal goat serum. The mouse polyclonal anti-nuclear factor kappa B (NF-κB), nitrotyrosine (NT), and inducible nitric oxide synthase (i-NOS) were used as primary antibodies. Then, the slide was incubated with biotin labeled IgG and horseradish peroxidase (HRP) conjugated streptavidin for 1 h, respectively. Immunoreaction was visualized employing diaminobenzidine and counterstained by hematoxylin. An image was taken by light microscopy.

2.8. Western blotting

Proteins were separated by 10% SDS-PAGE electrophoresis and transferred onto nitrocellulose membrane. After blocking with 10% skim milk, membranes were incubated with primary polyclone mouse antibodies CHOP, GRP78, ATF4, XBP1s and β-actin at room temperature for 3 h. After washing three times with PBS, membranes were further incubated with secondary antibody conjugated with HRP for 1.5 h. Finally, immune-reactive protein bands were detected by diaminobenzidine method. The relative density of protein expressions was quantitated by Image J software. Protein levels were standardized by comparison with β-actin.

2.9. Statistical analysis

Variables are expressed by mean and standard deviation. Data were presented as mean ± SE. Student's t-test (two groups) or one way ANOVA (multiple groups) were used. A p value < 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software (version 6.0, GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Luteolin was effective in preventing APAP-induced liver damage

To explore the protective effect of luteolin on APAP-induced hepatic toxicity, male C57BL/6 mice were orally administered luteolin or APAP to observe liver injury. First, we identified the optimal concentration of luteolin by serial concentrations (0–100 mg/Kg) for 1 day or 3 days. As in Fig. 1A–B, the treatment of 50 mg/kg daily for 3 days before APAP administration is proper. The concentration of APAP (300 mg/kg) is tested for our previous study. Serum ALT and AST were analyzed after 16 h of administration. Mice treated with APAP showed evidence of significant liver damage, as indicated by the notable increase of serum ALT and AST levels. ALT and AST were significantly increased in the APAP administered group compared to the normal control group ($p < 0.001$). However, luteolin administration significantly reduced serum ALT and AST levels (Fig. 1A–B). Meanwhile, the livers of APAP administered mice were more hemorrhagic than those of the control group, and the pretreatment of luteolin ameliorates the injury induced by APAP. This protective effect was further confirmed by analysis of H&E staining, as shown in Fig. 1C. Hepatocellular degeneration, sinusoidal congestion and hemorrhage, lymphocyte infiltration, lipid droplets formation and centrilobular necrosis were seen in the model group. After administration of luteolin for 3 days, the severity of liver injury was reversed, and the necrocytosis induced by acetaminophen was significantly alleviated (Fig. 1D–E), indicating that luteolin can protect the liver from acetaminophen-induced hepatotoxicity.

3.2. Luteolin was effective in inhibition oxidative stress

Lipid peroxidation has been reported to be closely related to APAP-induced toxicity [16]. The content of MDA was detected at 16 h after APAP treatment. Low levels of MDA were observed in the control