Journal of Ethnopharmacology 176 (2015) 321–326

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Evaluation of kudzu root extract-induced hepatotoxicity

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ARTICLE INFO

Article history:
Received 27 April 2015
Received in revised form
15 July 2015
Accepted 2 November 2015
Available online 9 November 2015

Keywords:
Kudzu root extract
Puerarin
Hepatotoxicity
Mice
HepG2 cells
Mice hepatocytes

ABSTRACT

Ethnopharmacological relevance: Kudzu root, the root of *Pueraria lobata* (Willd.) Ohwi, has been used as food and medicine for centuries, but few studies indicate that kudzu root may cause liver damage.
Aim of study: We studied the hepatotoxicity of kudzu root extract in mice, HepG2 cells and mice hepatocytes.
Materials and methods: Mice were administrated with kudzu root extract (10 mg/day) for 4 weeks, and then the biochemical analysis and histopathological changes were carried out. To explore the potential mechanism by which kudzu root extract-induced hepatotoxicity, HepG2 cells and mice hepatocytes were co-cultured with kudzu root extract or puerarin, which is a kudzu root isoflavone, for 2 h.
Results: The increase of serum ALT and AST and histopathological changes in treated mice revealed that kudzu root extract was hepatotoxic. The increase of LDH leakage for HepG2 cells and mice hepatocytes further confirmed hepatotoxicity of kudzu root extract. Kudzu root extract and puerarin significantly up-regulated *Mt1* mRNA involved in the acute phase response and *Bax* which is crucial for apoptosis. *Gclc*, *Nrf2* and *Ho-1* mRNA expressions did not change in treatment group.
Conclusions: Kudzu root extract may be hepatotoxic and caution may be required for its use.

1. Introduction

Herbal remedies have increased in popularity over time due to interest in alternative or complementary medicine. Herbal remedies have generally been regarded as safe because of their "natural" origin. However, many studies suggest that herbal compounds may seriously harm human health. For example, hepatotoxicity has been established for many herbal compounds (Lambert et al., 2010; Teschke et al., 2011a, 2011b). Therefore, evaluation of the safety of herbal preparations and their mechanism of action is needed to anticipate any potential toxicity.

Kudzu, *Pueraria lobata* (Willd.) Ohwi, that belongs to the family Leguminosae and the genus Pueraria is a perennial deciduous vine with hairy rusty-brown stems. Leaves are arranged alternately along the stem with three leaflets. The showy reddish-purple flowers have violet-purple to reddish-purple standards with yellow spots at the base. The seed pods are relatively flat, hairy, and mature in early fall. The compressed kidney-shaped seeds are nearly round and about 3–4 nm long. It can form underground tubers up to 2 m long at a depth of 1–5 m (Mitich, 2000; Wong et al., 2011). The active constituents of the plant mainly include isoflavonoids, triterpenoid saponins, chalcones, coumarins (Prasain et al., 2012). Its root has been commonly used as food and medicine for many centuries in China (Prasain et al., 2012). Kudzu root, the root of *Pueraria lobata* (Willd.) Ohwi, has been used for the treatment of cardiovascular diseases and diabetes and is reported to contain bioactive substances, such as daidzein, puerarin and daidzin (Wu et al., 2013; Yuan et al., 2014). In addition, kudzu root has been reported to have anti-oxidant, anti-hypertensive activity and anti-inflammatory activity in select mouse models (Bebrevska et al., 2010; Cai et al., 2011; Zhang et al., 2013).

In contrast, kudzu root extract-induced hepatotoxicity has been documented (Santosh et al., 2010; Shukla, 1995). Santosh's group reported that higher dosing of kudzu root extract (100–400 mg/100 g body weight) or its continuous use for 30 days (5–100 mg/100 g body weight) is hepatotoxic to mice. However, cellular and molecular mechanisms of kudzu root extract-induced hepatotoxicity remain poorly

Abbreviations: *Acat2*, cholesterolacyl transferase 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; *Bax*, Bcl-2-associated x protein; BUN, blood urea nitrogen; CREA, creatinine; DBIL, direct bilirubin; DMEM, Dulbecco's modified eagle medium; *Gclc*, glutamate-cysteine ligase catalytic subunit; GLU, glucose; *Ho-1*, heme oxygenase 1; LDH, lactate dehydrogenase; LDL, low density lipoprotein; mRNA, messenger RNA; *Mt*, metallothionein; *Mt1*, metallothionein-1; *Nrf2*, nuclear factor E2-related factor 2; PBS, phosphate buffer saline; *Pcsk9*, proprotein convertase subtilisin/kexin type 9; RT-PCR, reverse transcription polymerase chain reaction; TBIL, total bilirubin; TC, total cholesterol; TG, triglycerides; UA, uric acid

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http://dx.doi.org/10.1016/j.jep.2015.11.005
0378-8741/

understood. Therefore, we investigated the effect of kudzu root extract on normal mice, measuring biochemical parameters in blood and observing histopathological changes. Furthermore, HepG2 cells and mice hepatocytes were co-incubated with kudzu root extract or puerarin for 2 h to elucidate the underlying mechanisms. LDH assay was used to evaluate the cytotoxicity of kudzu root extract or puerarin. Finally, the effect of kudzu root extract or puerarin on mRNA expression of certain key genes was measured.

2. Materials and methods

2.1. Materials and chemicals

The kudzu, *Pueraria lobata* (Willd.) Ohwi, was purchased from Chinese herbal stores in the Jiangxi province and was identified macro- and microscopically according to the Chinese Pharmacopoeia. A voucher specimen (No. 20131109) of the kudzu was deposited in the Jiangxi University of Traditional Chinese Medicine. Kudzu root, the root of *Pueraria lobata* (Willd.) Ohwi, extract in the powder form was purchased from Jang Xi Wan Zai Qiannian Food Co., Ltd. (Yichun, China). Puerarin was obtained from Re Young Co., Ltd. (Linyi, China). All other chemicals and reagents used were analytical grade and obtained from local sources.

2.2. Animals and treatment

We purchased 20 BALB/c female mice (18 ± 2 g) from the laboratory animal science institute of Nan Chang University. Mice were housed in stainless steel cages (20 ± 2 °C room temperature; $50 \pm 5\%$ relative humidity) with a natural light/dark cycle. Mice had free access to standard laboratory feed and tap water. Animal handling and procedures were performed according to the ethical guidelines of Nan Chang University (Certificate No. SYXK-Gan-2010-0002). After mice acclimatized to the experimental facility for 1 week, the mice were randomly divided into two groups ($N=10$): (1) control group treated with 100 μL sterile PBS (0.01 M); (2) experimental group treated with 100 μL kudzu root extract solution (100 mg/mL). All mice were administrated by oral gavage, daily for 28 days.

2.3. Biochemical analysis

The biochemical analysis was performed to evaluate the effect of kudzu root extract on the biochemical parameters in blood (Morgan et al., 2014) with some modifications. After the experimental period, the mice were anaesthetized with diethyl ether by intraperitoneal injection (3 mL/kg) and sacrificed. Blood samples were collected from retro-orbital venous plexus for biochemical analysis. Blood samples were kept at room temperature for 2 h and then at 4 °C overnight. Blood samples were centrifuged at 3000 rpm for 10 min for serum separation. All biochemical parameters were measured with a PUZS–300 automatic biochemical analyzer in the first affiliated hospital of Nan Chang university. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL) and direct bilirubin (DBIL) were used to evaluate liver function. Creatinine (CREA), blood urea nitrogen (BUN) and uric acid (UA) were used to evaluate renal function. Glucose (GLU), triglycerides (TG), and total cholesterol (TC) were also measured.

2.4. Histopathological evaluation

Histopathological evaluation was performed to study the effect of kudzu root extract on liver function (Aycan et al., 2014; Salminen et al., 2012) with some modifications. After mice were sacrificed, liver tissues were carefully dissected out, washed with ice cold saline, and fixed into 10% formalin solution for 48 h. Then, fixed liver tissues were embedded in paraffin wax, sliced at approximately 5 μm in thickness, and stained with hematoxylin-eosin for histopathological evaluation. The sections were examined under a light microscope (Nikon, Japan) and scored by a liver pathologist. Hepatic injury was graded as follows: grade 0: absent; grade 1: necrosis of less than 6% of hepatocytes; grade 2: necrosis of 6–25% of hepatocytes; grade 3: necrosis of 26–50% of hepatocytes; grade 4: greater than 50% of hepatocytes.

2.5. Hepatocyte isolation and culture

The isolation and primary culture of mice hepatocytes methods were performed as described previously (Wei et al., 2009) with some modifications. After mice were sacrificed, hepatic tissues were carefully excised and washed with sterile PBS (0.01 M) for 3 times. The tissues were dissected into small pieces followed by collagenase IV digestion at room temperature for 30 min on a shaker (0.1% collagenase in PBS, pH 7.4, without Ca^{2+}). Then hepatic tissues were gently triturated by pipetting with a narrow tip and filtered through 70 μm nylon mesh. Cell suspensions were then centrifuged at 1000 rpm for 10 min. After the supernatant was discarded, cells were re-suspended in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin.

Hepatocytes (1×10^6 cells/flasks) were seeded in 25 cm^2 culture flasks in a humidified atmosphere (95% O_2; 5% CO_2) at 37 °C for 24 h to allow cell attachment. After cell attachment, 3 culture conditions were used: (1) untreated cultures; (2) cultures with 20 μL of kudzu root extract solution added (1 mg/mL); (3) cultures with 20 μL of puerarin solution added (0.02 mg/mL). Culture flasks were incubated for 2 h at 37 °C under 5% CO_2 in an incubator. Next, the supernatant was transferred into a new microcentrifuge tube and used at once or stored at −20 °C. Finally, 1 mL of Trizol reagent was added to culture flasks and used at once or stored at −80 °C.

2.6. HepG2 Cell culture and treatment

HepG2 (hepatocellular carcinoma cell) cells were cultured according to a previously described method (Souza et al., 2013) with some modifications. HepG2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin in a humidified atmosphere (95% O_2; 5% CO_2) at 37 °C.

HepG2 cells were seeded in 25 cm^2 culture flasks (2×10^5 cells/flask) and incubated for 24 h at 37 °C. Then, 20 μL of kudzu root extract solution (1 mg/mL), 20 μL of puerarin solution (0.02 mg/mL) and 20 μL of sterile PBS (0.01 M) were added in 3 culture flasks, respectively. After that, the cultured flasks were incubated for 2 h at 37 °C under 5% CO_2 in an incubator. The supernatant was transferred into a new centrifuge tube and used at once or stored at −20 °C until required. After that, 1 mL of Trizol reagent were added to culture flasks and used at once or stored at −80 °C.

2.7. LDH cytotoxicity

A lactate dehydrogenase (LDH) leakage assay was carried out to investigate the cytotoxicity of kudzu root extract or puerarin (Wesam et al., 2013) with some modifications. After HepG2 cells or mice hepatocytes were treated, 100 μL/well supernatant was transferred into a 96-well plate and incubated for 10 min at 37 °C. Then, 100 μL of the reaction mixture was added to each well and kept in the dark at room temperature for 15 min. Finally, 30 μL of citric acid solution was added to each well to terminate the reaction. The absorbance of all samples was measured at 570 nm.