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Artificial sweetener neohesperidin dihydrochalcone showed antioxidative, anti-inflammatory and anti-apoptosis effects against paraquat-induced liver injury in mice

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ABSTRACT

The present study evaluated the protective effect of artificial sweetener neohesperidin dihydrochalcone (NHDC) against paraquat (PQ)-induced acute liver injury in mice. A single dose of PQ (75 mg/kg body weight, i.p.) induced acute liver toxicity with the evidences of increased liver damage biomarkers, aspartate transaminase (AST) and alanine transaminase (ALT) activities in serum. Consistently, PQ decreased the antioxidant capacity by reducing glutathione peroxidase (GP-X), glutathione-S-transferase (GST) and catalase (CAT) activities, glutathione (GSH) level and total antioxidant capacity (T-AOC), as well as increasing reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) levels. Histopathological examination revealed that PQ induced numerous changes in the liver tissues. Immunochemical staining assay indicated the upregulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions. However, NHDC ameliorates PQ-induced hepatic toxicity in mice by reversing these parameters. Additionally, NHDC significantly inhibited PQ-induced nuclear factor-kappa B (NF-κB) expression and mitochondrial-driven apoptotic signaling. TUNEL assay confirmed that PQ-induced apoptosis was relieved by NHDC. In conclusion, these findings suggested that NHDC showed potent antioxidant, anti-inflammatory and anti-apoptotic effects against PQ-induced acute liver damage.

1. Introduction

Paraquat (PQ) is one of the most extensively used herbicides, especially in the developing countries [1]. There are thousands of case reports regarding PQ poisonings of human beings from accidental or intentional ingestions yearly [2]. Due to the lack of antidote or the effective strategy against PQ-induced toxicity, PQ has been classified as moderately hazardous material by the World Health Organization (WHO) [3].

The PQ's toxicity is mainly mediated by its redox activity. Multiple enzymes link in the metabolism of PQ, e.g., cytochrome P450 reductase, ferricytochrome oxidoreductase and nitric oxide synthase [4]. Primarily, PQ is reduced by NADPH cytochrome P450 reductase to monocationic $PQ^{\bullet+}$ radical by accepting an electron [5]. Consequently, this unpaired electron translocates to oxygen with the production of superoxide, while $PQ^{\bullet+}$ becomes PQ^{2+}. This process triggers a free radical cascade reaction, including the formation of hydrogen peroxide and hydroxyl radical, which plays an essential role on the PQ's cytotoxicity [6,7].

Previous studies illustrate that oxidative stress represents a predominant mechanism of PQ's toxicity in different experimental models, including plants, bacteria and animals [8]. Thus, the strategies of using different antioxidants against PQ-insult have been developed, such as the prevention of generation/distribution of ROS and the maintenance of effective levels of endogenous antioxidants [8,9].

Chalcones and their derivatives have been widely utilized for the development of chemopreventive agents due to their antioxidant and anti-inflammatory capacities [10]. Neohesperidin dihydrochalcone (NHDC) is a semi-synthetic glycoside chalcone, which is produced by catalytic hydrogenation of neohesperidin, the main flavanone glycoside of bitter oranges. NHDC has wide application in various foods and beverages as a low caloric artificial sweetener, however, its chemopreventive effect has not been identified until our recent studies suggest its protective antioxidant effect [11,12].

The main organs for PQ-distribution are lung and kidney [8]. Besides, PQ has a similar structure compared with 1-methyl-4-penylpyridinium

Abbreviations: ALT, alanine transaminase; ASK1, apoptosis signal-regulating kinase 1; AST, aspartate transaminase; Bcl-2, B-cell lymphoma-2; CAT, catalase; COX-2, cyclooxygenase-2; DCFH-DA, 2′, 7′-dichlorodihydrofluorescein diacetate; GP-X, glutathione peroxidase; GSH, glutathione; GST, glutathione S-transferase; IκBα, inhibitor of NF-κBα; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; JNK, c-Jun. N-terminal kinase; NHDC, neohesperidin dihydrochalcone; PQ, paraquat; ROS, reactive oxygen species; T-AOC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-alpha; TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling.

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ion (MPP^+), the metabolite of a dopaminergic neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP), which suggests PQ's neurotoxicity [13]. Due to these two reasons, most of the previous studies focus on lung, kidney and brain regarding PQ's toxicity. As a consequence, there is little information about the toxic effect of PQ on other tissues. Liver is the primary organ for xenobiotics detoxification, and indeed, a small group of researches confirm that the liver is a target of PQ [14]. However, how to combat with PQ-induced liver damage needs further investigation. Therefore, the present study was designed to explore the possible protective role of NHDC against PQ-induced hepatic injury, oxidative damage, inflammation and mitochondrial dysfunction in mice.

2. Materials and methods

2.1. Chemicals

NHDC (CAS number, 20,702-77-6) was purchased from Aladdin Chemistry Co. Ltd. PQ was purchased from TCI (Shanghai, China). Diagnostic kits used for the determination of aspartate transaminase (AST), alanine transaminase (ALT), glutathione peroxidase (GP-X), reduced glutathione (GSH), glutathione-S-transferase (GST) activities, catalase (CAT), total antioxidant capacity (T-AOC) and thiobarbituric acid reactive substances (TBARS) were obtained from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Rabbit IL-6, IL-1β, NF-κB (p65), Bax, caspase 9, caspase 3, Lamin B, β-actin, mouse TNF-α polyclonal primary antibodies and goat anti-mouse IgG-HRP-conjugated secondary antibody were obtained from Proteintech (Wuhan, China). Rabbit B-cell lymphoma-2 (Bcl-2), p-p65 polyclonal antibodies, goat anti-rabbit IgG-HRP-conjugated secondary antibody and total protein extraction kit were obtained from Sangon Biotech Co. Ltd. (Shanghai, China). Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), inhibitor of NF-κBα (IκBα), and p-IκBα antibodies were purchased from Bioss (Beijing, China). All other chemicals were of the highest grade commercially available.

2.2. Animals

Male Kunming mice weighing 18–22 g were used in all experiments. They were obtained from the Chongqing Academy of Chinese Medica. Animals were supplied with food and water ad libitum, and maintained in a standard temperature and humid environment with a 12 h light/dark cycle. All animal studies were approved by the Southwest University Animal Care and Use Committee. Experiments were conducted according to the NIH's guidelines for the care and use of laboratory animals.

2.3. Experimental protocol

In this experiment, mice were randomized into four groups with each group consisting of eight animals. The control group received equal volume of vehicles throughout. The PQ group received saline once daily for 6 consecutive days. One hour after final saline treatment, mice were injected with PQ (75 mg/kg body weight). The NHDC group received a daily dose of NHDC (200 mg/kg body weight, dissolved in a 0.5% CMC vehicle and prepared before use) by oral gavage for 6 consecutive days. NHDC + PQ group received a daily dose of NHDC (200 mg/kg body weight) by oral gavage for 6 consecutive days. One hour after final NHDC treatment, mice were injected with PQ (75 mg/kg body weight).

2.4. Sample collection

Animals were sacrificed 6 h after PQ administration. Blood samples were collected from the orbital plexus and centrifuged for biochemical parameter analysis. Livers were removed immediately, rinsed with ice-cold physiological saline and homogenized in saline to get a 10% homogenate. All steps were performed at 4 °C.

2.5. Biochemical parameters of liver function

Serums were separated by the centrifugation of blood at 600 *g* for 15 min. Serum AST and ALT activities were determined by using commercially available kits according to the manufacturer's recommended protocol (Jiancheng Institute, Nanjing, China).

2.6. Histopathological examination

Liver tissues were fixed in 4% paraformaldehyde. They were embedded in paraffin and sectioned for 5 μm thickness. After hematoxylin–eosin (H&E) staining, slides were observed for histopathological changes using fluorescent microscope system (TE2000, Nikon, Japan). Representative images were presented.

2.7. Determination of ROS level

ROS level was determined by using DCFH-DA as fluorescent probe. Fluorescence was measured in a HITACHI F7000 fluorescence spectrophotometer with an excitation wavelength of 480 nm and an emission wavelength of 538 nm.

2.8. Assays of oxidative biochemical parameter

Liver homogenates were used to determine the oxidative biochemical parameters. The GSH content, T-AOC and CAT/GST/GP-X activities were measured using commercial assay kits (Jiancheng Institute, Nanjing, China).

2.9. Western blotting assay

Total proteins or nuclear proteins were extracted by using commercially available kits according to the manufacturer's recommended protocol (Jiancheng Institute, Nanjing, China). Samples were separated on 10% or 12.5% SDS-PAGE, transferred onto nitrocellulose membranes and blocked in 5% BSA or 5% nonfat dry milk at 37 °C for 1.5 h. Then, the membranes were incubated with primary antibodies at 4 °C overnight. and appropriate secondary antibodies conjugated to horseradish peroxidase at room temperature for 2 h. The proteins were detected using the ECL system or the HRP substrate DAB system. β-Actin and Lamin B were used as loading controls.

2.10. Immunohistochemical staining

Paraffin-embedded liver sections were deparaffinized and rehydrated. Then, they were incubated with 3% H_2O_2 for 10 min and performed with a 1 mM EDTA buffer (pH = 9.0) in a microwave for 3 min. The following steps were performed according to the instructions of Histostain™-Plus and DAB substrate Kits. The sections were incubated with COX-2 or iNOS antibodies. After washing with PBS, sections were incubated with IgG-HRP-conjugated antibodies at room temperature for 1 h. Images were taken by a light microscopy (magnification, 400×, Nikon Eclipse Ti-SR).

2.11. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

Paraffin-embedded sections were deparaffinized and rehydrated. Tissue sections were incubated with biotinylated nucleotide mix, recombinant terminal deoxynucleotidyl transferase and equilibration at 37 °C for 1 h. After incubation, sections were immersed in 0.3% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase, and incubated with peroxidase solution at room temperature for