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# Puerarin protects mouse liver against nickel-induced oxidative stress and inflammation associated with the TLR4/p38/CREB pathway

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ABSTRACT

Nickel (Ni), one of hazardous environmental chemicals, is known to cause liver injury. Accumulating evidence showed that puerarin (PU) possessed comprehensive biological effects. The purpose of the current study was to test the hypothesis that the puerarin protects against enhanced liver injury caused by Ni in mice. ICR mice received intraperitoneally nickel sulfate (20 mg/kg/body weight, daily) for 20 days, and puerarin (200 and 400 mg/kg/body weight) was applied before Ni exposure. The results indicated that puerarin markedly inhibited Ni-induced liver injury, which was characterized by decreased aminotransferase activities and inflammation. Puerarin also inhibited the oxidative stress and decreased the metallothionein (MT) levels. Puerarin decreased the level of pro-inflammatory cytokines TNF-α and IL-6 in livers. Puerarin significantly inhibited the TLR4 activation and p38 MAPK phosphorylation, which in turn inhibited NF-κB activity. Likewise, Ni-induced inflammatory responses were diminished by puerarin as observed by a remarkable reduction in the levels of phosphorylated CREB. Furthermore, puerarin also reduced inflammatory mediators such as cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) levels in livers. Data from this study suggested that the inhibition of Ni-induced oxidative stress and inflammatory responses by puerarin is due to its ability to modulate the TLR4/p38/CREB signaling pathway.



## 1. Introduction

Nickel (Ni), an occupational and environmental toxin, is widely used in industry. Many studies had been demonstrated that Ni could induce genotoxicity, cytotoxicity, immunotoxicity, cancerogenicity and mutagenicity in vitro and in vivo [1,2]. Human exposure to Ni occurs primarily by breathing air, drinking water, consuming food, or smoking tobacco containing Ni [2]. After entering the body, Ni penetrates all organs and accumulates in various tissues and causes tissue damage [3]. Previous reports from our laboratory had revealed that Ni caused DNA injury in liver and these effects were demonstrated to be associated with reactive oxygen species (ROS) formation and apoptosis [4].

Metallothioneins (MTs) are highly conserved, small molecular weight, cysteine-rich proteins. MTs have the capacity to bind both physiological and xenobiotic heavy metals through the thiol group of its cysteine residues, which represents nearly 30% of its amino acidic residues [5,6]. A growing body of evidence suggests that Ni exposure can increase metallothionein [7–9].

Toll-like receptors (TLRs) have been reported to play an essential role in the activation of innate immunity [10,11]. In this murine model, elements of the innate immune system such as Toll-like receptor 4 (TLR4) may play important roles in the establishment of the metal allergy [12,13]. Activated TLR4 regulates a variety of downstream targets, which in turn inhibited NF-κB activity and the inflammatory cytokines [10,11,14]. The p38 mitogen-activated protein kinases (MAPKs) pathway plays a pivotal role in communicating signals, which are responsible for the production of the pro-inflammatory cytokines and downstream signaling events related to inflammation. Blocking p38 MAPK strongly inhibited production of the major inflammatory cytokines (i.e. tumor necrosis factor-α and interleukin-1) and other proteins (e.g.

*Abbreviations:* ALT, alanine aminotransferase; AST, aspartate aminotransferase; CREB, cAMP-responsive element binding protein; COX-2, cyclooxygenase-2; IL-6, interleukin-6; NF-κB, nuclear factor-κB; Ni, nickel; ROS, reactive oxygen species; PGE2, prostaglandin E2; TBARS, thiobarbituric acid reactive substances; TLR4, the Toll-like receptor 4; TNF-α, tumor necrosis factor-alpha.

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cyclooxygenase-2) [15]. Many researchers revealed that Ni-induced inflammation was associated with the TLR4/p38/NF-κB pathway [10,11,16]. Signaling through the p38 MAPK pathway also results in the downstream activation of cAMP-responsive element binding protein (CREB), which up-regulates expression of multiple inflammation-related genes [17,18].

Kudzu root (the root of *Pueraria lobata*), which has recently become commercially available in Western dietary supplements, is widely used in traditional Chinese medicine [19]. Puerarin (4′,7-dihydroxy-8-β-D- glucosylisoflavone, PU) is a major isoflavone compound isolated from the root of *Pueraria lobata* [20,21]. Several studies have shown that puerarin exerts anti-oxidative, anti-inflammatory, anti-apoptosis, cholesterol-lowering, hepatoprotective, renoprotective and neuroprotective effects [20–22]. A number of studies illustrated that puerarin can protect liver from injury induced by hepatotoxins [20,23,24]. Despite those pharmacological benefits, the molecular mechanisms underlying hepatoprotective effects of puerarin are still unclear.

In this study, we, for the first time, determine whether puerarin can protect mouse liver from Ni-induced oxidative stress and inflammation associated with the TLR4/p38 and CREB/NF-κB pathway.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Puerarin (>98% purity) and nickel sulfate ($NiSO_4$) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); TLR4 antibody, phospho-p38 antibody, p38 antibody, phospho-CREB(Ser133) antibody, NF-κB p65 antibody, TNF-α antibody, IL-6 antibody, COX-2 and MT antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Cell Signaling Technology (Beverly, MA, USA); Reagents and kits used in the assays of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); BCA assay kit from Pierce Biotechnology, Inc. (Rockford, IL, USA). All other reagents unless indicated were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Animals and treatment

Male ICR mice (male, 4 weeks old weighing approximately 20 g) were purchased from the Branch of National Breeder Center of Rodents (Beijing). The mice were maintained under constant conditions (23 ± 1 °C and 60% humidity) and had free access to rodent food and tap water under 12 h light/dark schedule (lights on from 08:30 to 20:30 h).

After acclimatization to the laboratory conditions, the mice were randomly divided into five groups (ten mice in each). Group I: served as control (treated intraperitoneally with physiological saline for 20 days); Group II: animals received intraperitoneally nickel sulfate (20 mg/kg/body weight, daily) following a daily oral gavage administration of physiological saline; Group III and IV: animals received intraperitoneally nickel sulfate (20 mg/kg/body weight, daily) following a daily oral gavage administration of puerarin (200 and 400 mg/kg body-weight, dissolved in physiological saline); Group V: animals received intraperitoneally physiological saline following a daily oral gavage administration of puerarin (400 mg/kg body-weight, dissolved in physiological saline). The choice of nickel sulfate dose is based on previous findings [25] and indicates the level of toxic intake of Ni in occupationally exposure and some emergency [3]. The choice of puerarin dose is based on previous findings, which showed that puerarin has protective effects on tissue damage [20,26].

The experiment lasted for 20 days. At the end of treatment, mice were sacrificed and about 1 ml of blood samples were drawn by cardiac puncture with heparined tubes. The liver tissues were immediately collected for experiments and placed in ice-cold 0.9% NaCl solution to remove blood cells, blotted on filter paper. And then the removed liver was immediately collected for experiments or stored at −70 °C for later use.

The present research reported in this paper was conducted in accordance with the Chinese legislation and NIH publication on the use and care of laboratory animals and were approved by the respective university committees for animal experiments.

### 2.3. Measurement of serum aminotransferase activities

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were estimated spectrophotometrically using commercial diagnostic kits (Jiancheng Institute of Biotechnology, Nanjing, China) [4].

### 2.4. Assay of thiobarbituric acid reactive substances (TBARS) levels in liver

Tissue lipid peroxidation was measured by our previous method [21]. Reaction mixture was incubated with 0.6% TBA (w/v) for 1 h in boiling water bath. Pink color chromogen was extracted in butanol-pyridine solution (15:1) and read at 532 nm.

### 2.5. Western blot analyses

MT expression was detected by a modified Western blot protocol as previously described [27]. Routine western blot for other protein expressions was performed as described in our previous studies [4,21].

### 2.6. Statistic analysis

All statistical analyses were performed using the SPSS software, version 11.5. A one-way analysis of variance (ANOVA; $P < 0.05$) was used to determine significant differences between groups and the individual comparisons were obtained by Turkey's HSD post hoc test. Statistical significance was set at $P \leq 0.05$.

## 3. Results

### 3.1. Puerarin protects against Ni-induced hepatic dysfunction

In order to determine whether puerarin can attenuate the liver damage in the Ni-treated mouse, we measured the activities of serum ALT and AST (Fig. 1). In Ni-treated mice, the activities of serum ALT and AST markedly increased by 195% and 116% as compared with those of the controls, respectively ($P < 0.05$). Interestingly, treatment with low and high dose of puerarin in Ni-treated mice significantly decreased the activities of serum ALT and AST ($P < 0.05$). No significant difference of ALT and AST activities could be seen in the blood from the mice treated with puerarin only as compared with vehicle controls.

### 3.2. Puerarin inhibited Ni-induced oxidative stress in liver

Many studies suggested that the TBARS level might be indicators of oxidative stress. The results showed that puerarin could decrease Ni-induced TBARS level (Fig. 2). Ni treatment significantly increases hepatic TBARS level by 115% as compared with those of the controls ($P < 0.05$). However, the treatment with low and high dose of puerarin inhibited this elevation ($P < 0.05$) (Fig. 2A). No significant