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Ligustrazine attenuates elevated levels of indoxyl sulfate, kidney injury molecule-1 and clusterin in rats exposed to cadmium

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ABSTRACT

In this study, we aimed at evaluating the effect of ligustrazine, a major constituent of *Ligusticum wallichii* from traditional Chinese medicine, on Cd-induced changes in nephrotoxicity indices. Rats were divided into four experimental groups: control; ligustrazine; Cd and ligustrazine + Cd. Cd treated alone group showed significant decreases ($P < 0.05$) in body weight, renal levels of superoxide dismutase (SOD) and glutathione reductase (GR); and significant increases ($P < 0.05$) in urine volume (24 h), pH values, serum blood urea nitrogen (BUN), serum uric acid, kidney malondialdehyde (MDA), urinary total protein, urinary glucose, urinary lactate dehydrogenase (LDH) and urinary alkaline phosphatase (ALP). Apart from indoxyl sulfate (a uremic toxin), two newly accepted nephrotoxicity biomarkers including kidney injury molecule-1 (kim-1) and clusterin were also found to be increased. Nonetheless, all these effects induced by Cd were reversed upon treatment by ligustrazine although it failed in decreasing the concentrations of Cd in kidney and urine. Histopathological studies in Cd-treated rats exhibited renal tubule damage, which was also ameliorated by ligustrazine pretreatment. These results suggest that ligustrazine exhibits protective effects on Cd-induced nephrotoxicity. Additionally, this study also demonstrates Cd exposure induces elevated levels of indoxyl sulfate in serum and kidney, and clusterin in urine.

1. Introduction

Cadmium (Cd), a well-known heavy metal, has multi-biological toxicities on multiple organ systems, especially in kidneys (Friberg, 1984; Edwards and Prozialeck, 2009; Nogawa and Kido, 1993; Prozialeck et al., 2007, 2009). Daily exposure to Cd, such as working in industrial workplaces, food, water and cigarette, are the main sources of Cd exposure for humans (Waisberg et al., 2003). Due to its low permissible exposure limit, overexposures may occur even in situations where small trace quantities of Cd are found having a negative effect on humans. For example, renal tubular damage can appear due to small traces of Cd. Moreover, Cd has a long biological half life ($t_{1/2}$) of 15 years in kidney and liver which extends its effect on the human body for a considerable period of time (Nordberg and Nishiyama, 1972). Thus, people pay more and more attention to Cd-induced nephrotoxicity in recent years.

Up to now, nephrotoxicity induced by Cd has been extensively studied and widely reported in occupationally and environmentally exposed human subjects (Renugadevi and Prabu, 2009), and more and more evidences demonstrated that Cd involves renal damage by reactive oxygen species (ROS), lipid metabolism disorder, glutathione depletion, protein cross-linking, DNA damage and oxidant-induced cell death ultimately (Renugadevi and Prabu, 2010; Amara et al., 2008; Abdel-Aziem et al., 2011; Ahammed et al., 2013; Conterato et al., 2013). Urinary excretion of some nephrotoxic biomarkers such as kidney injury molecule-1 (kim-1) (Prozialeck et al., 2007, 2009; Pennemans et al., 2011), glutathione S transferases alpha (GST-<alpha>) (Won et al., 2011), <beta> 2-microglobulin (Nakajima et al., 2005; Lei et al., 2012) were also found to be increased in Cd-induced nephrotoxicity. In view of the mechanism of Cd-induced nephrotoxicity, it is important to find a compound, which shows good functions on antioxidation, anti-cellular damage and so on.

Ligustrazine is the major constituent of *Ligusticum wallichii*, which has been used for treating cardiovascular disease, headache and vertigo for hundred years in Chinese medicine (Li, 2003). Ligustrazine exhibits a strong anti-oxidative activity, which is attributed to the pyrazine skeleton in the structure of ligustrazine and its metabolites in *vivo* (Liu et al., 2003). Several reports have demonstrated that ligustrazine showed an ameliorated effect on drug (cisplatin)-induced acute nephrotoxicity (Ali et al., 2008; Liu et al., 2008).

However, different kinds of renal damage exhibit various forms of clinical symptoms due to different mechanisms. For example, cisplatin-induced nephrotoxicity characterized by the glomerular dysfunction appeared earlier than tubular damage, whereas Cd-in-

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duced nephrotoxicity appeared by the reverse order (Wang and Wang, 1996). So it is unclear whether ligustrazine has the protective effect on heavy metals (HMs)-induced chronic nephrotoxicity characterized by renal tubule damage (Edwards and Prozialeck, 2009). Furthermore, little information is available on the roles of indoxyl sulfate, a uremic toxin aggravated the renal damage as an oxidative damage promoter (Shimizu et al., 2012) and clusterin, a novel urinary kidney biomarker (Hoffmann et al., 2010) in HMs-induced nephrotoxicity. Thus, the present study was designed to evaluate the protective effect of ligustrazine, and the response of indoxyl sulfate and clusterin on Cd-induced nephrotoxicity characterized by renal tubule damage in rats.

2. Materials and methods

2.1. Chemicals and animals

Ligustrazine hydrochloride was bought from Weifang Fine Chemical Co. Ltd. (Shanghai, China). Cadmium chloride (purity >99.0%) and indoxyl sulfate (purity >99.0%) were bought from Sigma–Aldrich Biotechnology (St. Louis, MO, USA). Phosphate-Buffered Saline (PBS, pH = 7.4) was bought from Yuanye Biotechnology (Shanghai, China). Acetonitrile and water (HPLC grade) were provided by Sinopharm Chemical Reagent (Shenyang, China). Trifluoroacetic acid (TFA) was bought from Kermel Chemical Reagents (Tianjin, China). Polyvinylidene fluoride (PVDF) membrane and western horseradish peroxidase (HRP) substrate were obtained from Millipore (Bed-ford, MA, USA). Primary antibodies including goat polyclonal antibody against kim-1 provided by R&D system (Shanghai, China), mouse polyclonal antibody against clusterin provided by Santa Cruz (CA, USA). All the other reagents were analytical grade. Redistilled water, distilled water, prepared with purified water, was used throughout this study.

Male pathogen-free Sprague–Dawley rats (60–70 g) were bought from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). They were housed four rats per plastic cage with free access to food and water. Temperature (22 ± 2 °C) and humidity (range 50–60%) were controlled by a completely automatic device. The dark/light cycle was 12/12 h (light on at 7.00 am; light off at 7.00 pm). Animal experiments were carried out in accordance with the "Guidelines for Animal Experimentation of Shenyang Pharmaceutical University" and the protocol was approved by the Animal Ethics Committee of this institution. The rats were fed with a standard laboratory diet and water for at least 1 week before the experiments.

2.2. Experimental design

All animals were randomly assigned into four groups containing eight rats per group. Rats were divided into four groups as described below. The volume injected of each dose was adjusted to 5 mL/kg per rat on the basis of the daily body weights of the rats just before dosing:

I: *Control group*. Rats were treated with saline by an i.p. injection at 2.00 pm and followed by an i.g. injection of that 1 h later per day for 5 times/week during six weeks.
II: *Ligustrazine group*. Rats were treated with ligustrazine (50 mg/kg) by an i.p. injection at 2.00 pm per day for 5 times/week during six weeks.
III: *Cd group*. Rats were exposed (i.g.) to 40 mg/kg $CdCl_2$ at 3.00 pm per day for 5 times/week during six weeks.
IV: *Ligustrazine + Cd group*. Rats were daily treated with ligustrazine by an i.p. injection at 2.00 pm and followed by an i.g. injection of $CdCl_2$ 1 h later in the same manner described in the "group II" and "group III", respectively; for 5 times/week during six weeks.

The body weight of each subject was recorded daily. After the final treatment, all rats were immediately kept in individual metabolic cages to collect urine for 24 h. After body weight, urine volume and pH were recorded, all urine samples were immediately stored at −80 °C in a freezer for the urinary biochemical parameters, novel kidney biomarkers and Cd analysis. Then, the rats were killed and the blood samples were collected from the arteria femoralis and centrifuged at 3000 rpm for 10 min, the serum obtained was stored at −20 °C freezer until the biochemical and indoxyl sulfate analysis was performed. Kidney tissues were excised and weighed immediately as well as each portion. A small portion of the left kidney was fixed in 10% neutral buffered formalin for histopathologic use, other portion of that was assigned to the analysis of indoxyl sulfate, superoxide dismutase (SOD), glutathione reductase (GR) and malondialdehyde (MDA). The right kidney was frozen in liquid nitrogen and then stored at −80 °C for the analysis of Cd.

2.3. Analysis of biochemical parameters, antioxidant enzymes and indoxyl sulfate

Serum biochemical parameters including blood urea nitrogen (BUN), creatinine, uric acid, and glucose were analyzed using an automated Hitachi Analyzer (Hitachi Medical Corporation, Tokyo, Japan). Urinary biochemical parameters included total protein creatinine, glucose, alkaline phosphatase (ALP), lactic dehydrogenase (LDH). Among them, urinary protein was determined by the protein assay reagent (Bio-Rad, Hercules, CA, USA); creatinine, glucose, ALP and LDH were determined using a urine chemistry analyzer (TBA-200FR NEO, Toshiba, Japan). Kidney SOD, GR and MDA were measured by commercial kits from EIAab Science (Wuhan, CN), Lianshuo Biological (Shanghai, CN) and Jiancheng Bioengineering Institute (Nanjing, CN), respectively.

Also, indoxyl sulfate levels in serum and kidney were determined chromatographically using a LC-10ATVP series HPLC system (Shimadzu, Tokyo, Japan) (Hou et al., 2008). Each serum sample (200 μL) was extracted for 3 min with 600μL acetonitrile. After centrifugation (12,000 rpm, 5 min), the organic phase was transferred to another vial and evaporated to dryness at room temperature under a slight stream of nitrogen, the serum sample residue was subsequently reconstituted with 200 μL of mobile phase. Kidney homogenate was made by spiking the same amount of physiological saline as the kidney's weight and treated with the same procedure as the serum samples.

The separation of indoxyl sulfate was performed on a Zorbax Eclipse plus C_{18} column (250 × 4.6 mm i.d., 5 μm particle size, Agilent, CA, USA) protected by an Octadecylsilyl (ODS) guard column (5 × 4.6 mm i.d., 5 μm particle size, Agilent, CA, USA), using acetonitrile: water containing 0.1% TFA (7.5:92.5, *v*/*v*) as mobile phase at a flow rate of 0.8 mL/min at room temperature. The wavelength was set to 280 nm and the injection volume to 20 μL for the kidney and the serum samples.

2.4. Analysis of kidney and urinary Cd

Kidney and urinary levels of Cd were analyzed according to the routine protocol (Horng et al., 2002) but with a slight methodological change for kidney Cd analysis, using a Varian Spectro-AA30 atomic absorption spectrophotometer (Spectro, Kleve, Germany).

2.5. Histological examination

For the hematoxylin and eosin (H&E) staining, several consecutive paraffin sections were de-paraffinized with xylene for 7 min (3 times). After gradual rehydration in a series of graded alcohols and after washing them with deionized water, the sections were stained with H&E for 1 min, rinsed with deionized water and developed in tap water for 5 min. The tissue sections were destained by dipping the slide in acidified ethanol and rinsing in tap water. After washing them with deionized water, the sections were stained with eosin for 30 s, dehydrated, and mounted. The histopathological findings were determined using an Axio Scope.A1 photonic microscope (Carl Zeiss, Oberkochen, Germany).

2.6. Urinary excretion analysis of kim-1 and clusterin

For determining the excretion levels of novel urinary biomarkers which are associated with kidney tubule damage, kim-1 and clusterin were selected to evaluate the tubule damage by western-blot. The urine samples (200 μL) were centrifugated for 5 min at a speed of 3000 rpm. After that, all samples were diluted with the same amounts of PBS, and collected to determine the protein concentration. The concentration of urinary proteins was measured and normalized spectrophotometrically in a 260 UV spectrophotometer (Shimadzu, Tokyo, Japan) using a protein assay (Bio-Rad Laboratories, Hercules, CA) based on the method described by Bradford (1976), and then all urine samples were denatured by boiling in 5× sample loading buffer (250 mM Tris–HCl, pH6.8, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5% β-mercaptoethanol) at 96 °C for 3 min. After the resolution of 15 μg protein by 8–15% SDS–PAGE using Power Pac Basic electrophoresis apparatus (Bio-Rad, Hercules, CA, USA), protein samples were electrophoretically transferred onto PVDF membranes, respectively. The membranes were blocked with 5% skim milk for 1 h and subsequently incubated with primary and secondary antibodies. Primary antibodies included goat polyclonal antibody against kim-1 (1:1000), mouse polyclonal antibody against clusterin (1:1000). Reactivity was detected using anti-goat and anti-mouse HRP-linked secondary antibody (1:10000). Immunoreactive bands were visualized via the ECL Plus western blotting detection reagents and quantified via Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). The experiments were repeated at least four times.

2.7. Statistical analysis

Values are expressed as a mean ± standard error of mean (SE) ($n = 8$). Differences between the means of the four groups were estimated by a one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison tests. A value of $P < 0.05$ was considered statistically significant.