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Danshen Modulates Nrf2-mediated Signaling Pathway in Cisplatin-induced Renal Injury*

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Summary: Danshen, an efficacious agent for cardiovascular diseases, has been found to play an essential role in kidney injury. In the present study, the effect of Danshen on cisplatin-induced renal dysfunction was investigated in a mouse model. Danshen was administered to mice at a dose of 3 g/kg 4 days before and 3 days after cisplatin treatment. A single intraperitoneal injection of 20 mg/kg cisplatin was used to induce nephrotoxicity. The mice were sacrificed 72 h after cisplatin intoxication. Biochemical parameters including serum creatinine and blood urea nitrogen were analyzed. Histopathological changes of kidney tissues were detected using HE staining. Antioxidant enzymes (GSH-Px and SOD) and peroxidative product (MDA) were detected. Protein expressions of Nrf2 and its target genes including HO-1 and NQO1 were measured by Western blotting. The results showed that pretreatment with Danshen significantly reduced serum creatinine and blood urea nitrogen in the cisplatin-treated mice. Histopathological examination showed that Danshen mitigated the renal damage induced by cisplatin. Moreover, Danshen restored the activities of antioxidant enzymes (GSH-Px and SOD) and normalized the MDA contents in renal tissues. Western blotting revealed that Danshen enhanced the expressions of Nrf2 and its target genes in cisplatin-exposed mice. It was suggested that Danshen protects against the cisplatin-induced renal impairment in the mice, which is potentially associated with the upregulation of Nrf2-mediated signaling pathway.
Key words: cisplatin; nephrotoxicity; Danshen; Nrf2; oxidative stress

Cisplatin is the first generation of platinum-based antineoplastic agent and has been widely used to treat various solid tumors, such as non-small cell lung cancer, ovarian cancer and prostate cancer. However, high-dose or long-term medication with cisplatin is limited by frequent occurrences of renal toxicity[1, 2]. The kidney, particularly the S3 segment of the proximal tubule, accumulates cisplatin preferentially, causing some mild reversible or irreversible renal damages, which are noticed in 20%–30% of patients undergoing cancer chemotherapy with cisplatin[3]. Nephrotoxicity caused by cisplatin is clinically manifested as a decline in the glomerular filtration rate (GFR), increases in serum creatinine (SCr) and blood urea nitrogen (BUN), and disturbances in the serum electrolytes[4, 5], severely affecting patients' quality of life. Currently, there is no effective therapy to prevent cisplatin-induced acute kidney injury. Further understanding of toxicity induced by cisplatin helps seek potential treatments.

Cellular and molecular mechanisms responsible for cisplatin-induced acute renal injury are not fully elucidated, but there is evidence that oxidative stress is involved in the pathological process[6–8]. It is generally accepted that the excessive superoxide radicals caused by cisplatin lead to the imbalance of antioxidant status and contribute to lipid peroxidation, while the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) exerts cytoprotective effect on the oxidative stress by enhancing antioxidant and detoxifying genes. Researchers concluded that Nrf2 absence exacerbated cisplatin-induced acute kidney injury and agents activating Nrf2 exerted protective roles[9, 10].

Danshen, a traditional Chinese herbal medicine, is prepared from the dry root of *Salvia miltiorrhiza Bunge*. Previously, the chemical fingerprint of Danshen for injection was characterized by high performance liquid chromatography (HPLC) and the major compositions were analyzed[11]. Injection of Danshen or its active components is recognized as effective in alleviating renal toxicity in recent studies[12–16], and positive outcomes also have been achieved in clinical practice[17]. However, to our knowledge, no studies have investigated the role of Danshen in the nephrotoxic model induced by cisplatin.

In the current study, we evaluated the therapeutic potential of Danshen against cisplatin-induced nephrotoxicity in an *in vivo* mouse model. In addition, the possible renoprotective mechanisms of Nrf2-mediated antioxidant pathway were investigated.

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1 MATERIALS AND METHODS

1.1 Chemicals

Danshen injection was obtained from Chiatai Qingchunbao Pharmaceutical Co., Ltd. (China). Cisplatin was purchased from Sigma-Aldrich (China). Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and malondialdehyde (MDA) kits were purchased from Jiancheng Biological Engineering Institute (China). Radioimmunoprecipitation assay (RIPA) buffer was purchased from APPLYGEN (China). Primary antibodies against HO-1 and NQO1 were from Abcam (USA), and primary antibodies against Nrf2 from Santa Cruz Biotechnology (USA). Secondary antibodies, HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were from Proteintech (China).

1.2 Animals

Male ICR mice, weighing 20–23 g, provided by the Experimental Animal Center of the Second Xiangya Hospital, were acclimatized for 1 week under standard condition (23±2°C, 12 h:12 h light/dark cycle) with free access to food and water. All experimental procedures were in line with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People' s Republic of China. Approval was obtained from the Ethics Committee of Central South University.

1.3 Experimental Design

Mice were randomly divided into 4 groups: control group, in which mice only received normal saline; Danshen group, in which mice were treated with Danshen (3 g/kg); cisplatin group, in which a single intraperitoneal injection of cisplatin (20 mg/kg) was administered; Danshen+cisplatin group, in which Danshen was intraperitoneally given at 3 g/kg 4 days before and 3 days after the cisplatin treatment. The dose of Danshen (3 g/kg) was chosen on the basis of our preliminary studies. The body weight was recorded throughout the experiment, and the drug dose was adjusted accordingly. On day 8, blood sample was collected. The mice were euthanized and their kidneys were removed. The left kidney was sectioned into blocks and immersed in 4% buffered paraformaldehyde solution to obtain histological sample and the right kidney was immediately transferred to liquid nitrogen and stored at −80°C for future molecular biological analysis.

1.4 Serum Markers of Kidney Damage

Blood samples were collected and SCr and BUN were determined with an automatic analyzer.

1.5 Histological Examination

Histopathological changes were assessed in 5 μm thick deparaffinized kidney tissue sections stained with hematoxylin and eosin (HE). Tubular damage in the kidney sections was examined at ×400 original magnification with a light microscope.

1.6 Determination of Renal GSH-Px, SOD and MDA

Each kidney tissue was homogenized with multifunctional homogenizer. The homogenate was centrifuged, and the supernatant was collected for biochemical analysis. The activities of GSH-Px and SOD, as well as MDA contents in the homogenate were determined with commercially available kits. The conditions and procedures followed the manufacturers' protocols.

1.7 Immunoblotting Analysis

The kidneys were lysed in RIPA buffer and centrifuged at 12 000 r/min for 15 min at 4°C. The protein concentrations were measured by the Bradford method. Volume equivalents of proteins were analyzed by 12% SDS-PAGE in each lane and transferred onto the PVDF membranes. After being blocked in 5% non-fat milk in TBST [25 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Tween 20] for 1 h at room temperature, the membranes were incubated with primary antibodies against Nrf2 (1:500), HO-1 (1:250) and NQO1 (1:1000) at 4°C overnight. The immunoblots were then incubated with a secondary antibody conjugated with horseradish peroxidase for 45 to 60 min. Signals were detected with an electrochemiluminescence (ECL) kit (Thermo Scientific/Pierce, USA). The intensity of each band was determined by Image J software (National Institutes of Health, USA). The signals were normalized to β-actin as the internal standard.

1.8 Statistical Analysis

Statistical Package for the Social Science (SPSS) version 18 (SPSS Inc., USA) was used. All values were expressed as $\bar{x}\pm s$, and analyzed with one-way analysis of variance (ANOVA), followed by Tukey's test. The level of significance was set at $P<0.05$.

2 RESULTS

2.1 Effect of Danshen on Cisplatin-induced Nephrotoxicity in ICR Mice

SCr and BUN levels were employed to determine renal function. As shown in table 1, cisplatin administration resulted in significant increase in the BUN and SCr, compared with those in the control group, providing evidence of renal injury. In contrast, this rise was prevented by Danshen supplementation. In addition, these serum biomarkers in the group with Danshen treatment alone didn't differ from those in the control group.

Table 1 Effect of Danshen on SCr and BUN in mice treated with cisplatin (n=6)

Groups	Creatitine (μmol·L^{-1})	Urea nitrogen (mmol·L^{-1})
Control	3.47±0.56	8.49±1.03
Danshen	3.46±0.86	8.07±1.24
Cisplatin	21.76±3.63***	42.49±6.22***
Danshen+cisplatin	11.88±3.84$^{\#\#\#}$	25.47±8.83$^{\#\#\#}$

$^{***}P<0.001$ *vs.* control group, $^{\#\#\#}P<0.001$ *vs.* cisplatin group

2.2 Effect of Danshen on Cisplatin-induced Histopathological Changes in ICR Mice

The morphological changes in the kidneys were histologically assessed by staining kidney specimens with HE. The kidney sections in the control mice and Danshen-treated mice exhibited normal architecture of renal tissues. Cisplatin treatment elicited intense necrotic and degenerative alterations which were markedly at-

tenuated by Danshen addition (fig. 1).

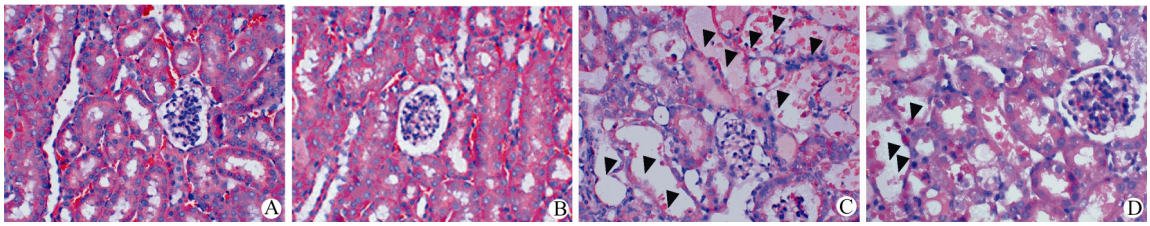

Fig. 1 Effect of Danshen on the histological changes induced by cisplatin in kidney tissues
Renal tissues obtained from treated mice were stained with HE. Tubular injury was observed under a light microscope (×400). A: control group; B: Danshen group; C: cisplatin group; D: cisplatin+Danshen group. Black triangles show necrotic and degenerative alterations.

2.3 Effect of Danshen on Cisplatin-induced Oxidative Damage in ICR Mice

Oxidative stress was quantified by measuring the activities of renal GSH-Px, SOD and contents of MDA in kidney homogenates (table 2). Significant reductions in the activities of GSH-Px, SOD and increases in MDA contents were found in the renal tissues of cisplatin-treated mice, indicating oxidative damage. In contrast, concomitant treatment with Danshen potentiated the renal antioxidant molecules and lowered the MDA levels to some extent. There was no difference between the control and Danshen-treated mice in the activities of antioxidant enzymes and MDA contents.

Table 2 Effects of Danshen on SOD, GSH-Px and MDA levels in mouse kidneys with or without cisplatin addition (*n*=6)

Groups	GSH-Px (U/mg protein)	SOD (U/mg protein)	MDA (nmol/mg protein)
Control	68.86±9.90	9.08±2.93	1.95±0.90
Danshen	58.27±11.79	8.62±2.61	2.40±0.94
Cisplatin	32.92±13.63***	4.75±1.33**	5.23±1.39***
Danshen+cisplatin	51.03±17.56$^{\#}$	7.81±2.42$^{\#}$	3.31±1.31$^{\#\#}$

$^{**}P$<0.01, $^{***}P$<0.001 *vs.* control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* cisplatin group

2.4 Effect of Danshen on the Protein Expression of Nrf2-mediated Signaling Pathway

To determine whether Danshen elicits its effects by regulating Nrf2-mediated signaling pathway, the expressions of Nrf2 and Nrf2-target genes HO-1 and NQO1 were detected by Western blotting. As presented in fig. 2, cisplatin treatment dramatically reduced the cytoprotective protein levels of Nrf2, HO-1 and NQO1, as compared with their expressions in the kidneys from the control group. In contrast, Danshen combination significantly promoted the expressions of these proteins negatively impacted by cisplatin. Compared with that in the control mice, the expression of Nrf2 in Danshen-treated mice was slightly elevated, but no statistical significance was noted.

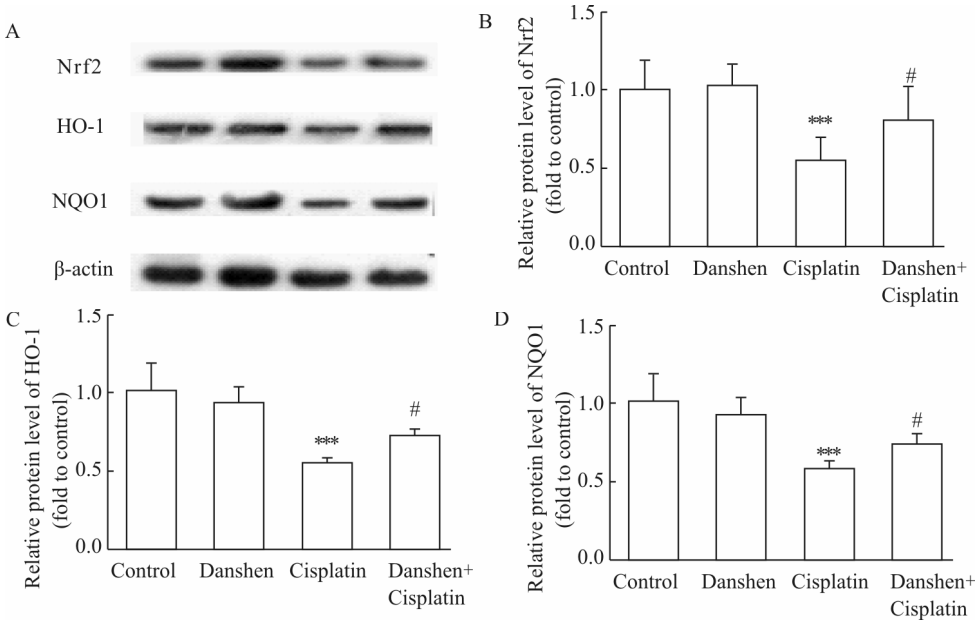

Fig. 2 Effect of Danshen on protein levels of Nrf2 and Nrf2-target genes in mice
A: Representative blots for Nrf2, HO-1 and NQO1 are shown. B–D: The density of the immunoreactive bands of Nrf2 (B), HO-1 (C) and NQO1 (D) was analyzed using β-actin as a control (*n*=6). $^{***}P$<0.001 *vs.* control group, $^{\#}P$<0.05 *vs.* cisplatin group

3 DISCUSSION

Danshen, one of the most popular traditional herbal medicine, is well known for its cardioprotective effect and is extensively used in China. Current evidence shows that Danshen may also have beneficial effects on kidney dysfunction due to its anti-oxidative and radical-scavenging capacity. However, few researches examined the effectiveness of Danshen as an adjuvant therapy for cisplatin-induced nephrotoxicity. The present study focused on the effect of Danshen on cisplatin-induced nephrotoxicity and the mechanism involving oxidative stress, which has been reported to be a plausible pathomechanism of cisplatin-induced renal functional alterations[18, 19].

Previous reports showed that cisplatin exposure led to obvious kidney injury manifested by increased SCr and BUN[7]. Moreover, the contents of a secondary product of lipid peroxidation, MDA, were elevated, and endogenous antioxidant enzymes GSH-Px and SOD, which are responsible for scavenging free radicals, were markedly suppressed by cisplatin in kidney tissues[18, 19]. Nrf2, a redox-sensitive transcription factor, has been verified to protect against many kidney dysfunctions by the induction of the expressions of cytoprotective genes, thus removing superoxide free radicals[20, 21]. Nrf2 activation initiates the production of drug-metabolizing enzymes, including HO-1, NQO1, glutamate-cysteine ligase (GSL), glutathione-S-transterase (GST), and acts synergistically with phase-Ⅲ membrane transporters such as multidrug resistance-associated proteins (MRPs)[22]. Among them, HO-1 and NQO1 activation is primarily liable for the alleviation of cisplatin-induced nephrotoxicity. HO-1 is a microsomal enzyme that catalyzes the degradation of soluble and toxic heme. The induction of HO-1 occurs as a protective response in cisplatin-induced renal tubular apoptosis, whereas HO-1 gene ablation reverses the protective effect, indicating the importance of this enzyme in protecting against cisplatin-induced nephropathy[23]. NQO1, a cytosolic antioxidant flavoprotein, catalyzes the 2-electron reduction of quinones to hydroquinones, causing detoxification of the electrophilic compounds and the prevention of redox cycling. Prior study[24] explained that NQO1 activation through β-lapachone increased the intracellular NAD^+ level and protects against acute kidney injury induced by cisplatin in wild-type mice rather than in $NQO1^{-/-}$ mice.

Moreover, Danshen, danshensu and salvianolic acid B are demonstrated to be Nrf2 inducers in various disease models[25−28]. Our research, however, showed that Danshen didn't induce the expression of Nrf2 in the kidney of mice distinctly, and this might be caused by the complicated components of Danshen injection investigated. In this regard, further studies are needed to ascertain whether the main active components, especially salvianolic acid B and danshensu, enhance the expression of Nrf2 in cisplatin-exposed mouse model. Recently, cryptotanshinone and Tanshinone ⅡA, active components in Danshen extracts, have been reported to display anti-proliferation and pro-apoptosis activities in multiple cancer cells[29], from which we infer that Danshen may have synergistic anticancer effect when co-administered with cisplatin in cancer patients. Therefore, further study is required to determine the effect of combined use of Danshen and cisplatin in tumor xenograft mouse models.

Collectively, for the first time, our study showed that Danshen attenuated cisplatin-induced nephrotoxicity in mice, which is possibly due to its modulating effect on Nrf2 signaling pathway. Our study may provide valuable reference for the use of Danshen in intervening renal toxicity after cisplatin administration in clinical practice.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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