Original Paper

The Protective Effects of Sika Deer Antler Protein on Cisplatin-Induced Nephrotoxicity

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Key Word

Oxidative stress • Apoptosis • Inflammation • Renal protection

Abstract

Background/Aims: This study measured the effect of Sika deer (*Cervus nippon* Temminck) antler protein (SDAPR), glycoproteins (SDAG), and polysaccharides (SDAPO) on cisplatin-induced cytotoxicity in HEK 293 cells, and investigated the effect of SDAPR against cisplatin-induced nephrotoxicity in mice. **Methods:** Cell viability was measured by MTT assay. ICR mice were randomly divided into five groups: control, cisplatin with vehicle, and cisplatin with SDAPR at three concentrations: 5, 10, or 20 mg/kg, p.o., 10 d. Cisplatin was injected on 7th day (25 mg/kg, i.p.). Renal function, oxidative stress, levels of inflammatory factors, and expression of apoptosis-related proteins were measured *in vivo*. Renal tissues were stained with TUNEL and H&E to observe renal cell apoptosis and pathological changes. **Results:** Pretreatment with SDAPR (125–2000 µg/mL) significantly improved cell viability, with an EC₅₀ of approximately 1000 µg/mL. SDAPR also ameliorated cisplatin-induced histopathologic changes, and decreased blood urea nitrogen (BUN) and creatinine (Cr) ($P < 0.05$). Western blotting analysis showed SDAPR clearly decreased expression levels of cleaved-caspase-3 and Bax, and increased the expression level of Bcl-2 ($P < 0.01$). Additionally, SDAPR markedly regulated oxidative stress markers and inflammatory cytokines ($P < 0.05$). TUNEL staining showed decreased apoptosis after SDAPR treatment ($P < 0.01$). **Conclusions:** These results indicate that SDAPR can be an effective dietary supplement, to relieve cisplatin-induced nephrotoxicity by improved antioxidase activity, suppressed inflammation, and inhibited apoptosis *in vivo*.

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Introduction

The World Cancer Report of 2014 identified a high incidence of cancer in China. Clinical trials have shown that the most common cancers include esophagus, lung, and stomach [1]. Currently, there are three types of therapies for cancers, surgery, radiation, and drug therapy. In medicinal treatment, platinum-based drugs are commonly used for cancer treatment. Platinum-based drugs accounted for 70-80% of antitumor chemotherapy in China [2], including cisplatin (CDDP), carboplatin (CBP), oxaliplatin (L-OHP), nedaplatin (NDP), and lobaplatin (LBP). Of these, CDDP is most common in clinical application [3].

Cisplatin (cis-diamminedichloroplatinum, CDDP) is an anticancer agent used in treating various solid tumors in the head, neck, lung, esophagus, bladder, and other parts of the body. [4]. However, there are many side effects of CDDP, including peripheral neurotoxicity, ototoxicity, myelosuppression, and nephrotoxicity [5]. Of these effects, the clinical limitation of CDDP is nephrotoxicity [6]. The clinical symptoms of CDDP nephrotoxicity are renal damage and dysfunction, and approximately 25-35% of patients showed signs of nephrotoxicity following a single dose of CDDP [7]. There are few effective remedies to repair renal structure and improving renal function [8]. Studies have indicated that the molecular mechanism of CDDP nephrotoxicity is related to oxidative stress [9], inflammation [10], and apoptosis [11]. Therefore, it is necessary to identify strategies to alleviate CDDP nephrotoxicity.

Velvet antlers are one of the most important animal medicines, and have been used for a variety of functions for more than two thousand years, including tissue repair, treatment of bone-resorption diseases, and treatment of rheumatoid arthritis [12]. Velvet antlers from Sika deer or red deer are deemed as medicinal antlers in national pharmacopoeias in China, Japan, and Korea [12]. In-depth studies have demonstrated the presence of mineral elements, polypeptides, proteins, amino acids, polysaccharides, and fatty acids in velvet antlers [13]. More recently, it has been reported that the aqueous extract of Sika deer antlers can protect against CDDP-induced nephrotoxicity in HEK 293 cells and mice [14]. In the present study, we explored which active ingredient of Sika deer antler aqueous extract can protect against CDDP nephrotoxicity and investigate the potential mechanisms of action.

Materials and Methods

Sample extraction

Samples of Sika deer antler powder (150 g) were purchased from Shuangyang deer farm (Changchun, China) and authenticated by Ph.D Zhang. The powder was put into 300 mL of water, and was extracted by ultrasound device (KQ5200DB, Kunshan, China) at 60°C for 0.5 h. The solution was filtered and collected, three times. The material was precipitated by addition of 95% (v/v) ethanol to a final concentration of 85% (v/v) and then maintained for 12h at 4°C. Next, the precipitate was collected by centrifugation (8000 rpm, 10 min). The filtrate was collected and the alcohol was removed using a rotary evaporator (R206, Shanghai, China). Then, the extracted solution was lyophilized in a freeze dryer (Telstar, Spain) to obtain the Sika deer antler protein powder (SDAPR). The precipitate was divided into two equal parts, and one part was lyophilized to obtain Sika deer antler glycoprotein (SDAG). Another part was treated to remove the proteins using the Sevage method and then lyophilized to obtain Sika deer antler polysaccharide (SDAPO).

Cell culture

Immortalized human embryonic kidney 293 cells (HEK 293) and human prostate cancer cells (PC-3M) were purchased from ATCC and stored at the Laboratory of Molecular Biology in Jilin Agricultural University, for use in the *in vitro* studies. The HEK 293 cells were cultured in DMEM (Hyclone, USA) and PC-3M cells were cultured in RPMI1640 (Hyclone, USA), both supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 1% penicillin/streptomycin (Hyclone, USA). The cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

MTT assay

The MTT assay, a quantitative colorimetric assay with 3-(4, 5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide, was used for determining cell viability. Cells were seeded into 96-well plates and cultured at 37°C for 24 h. After an appropriate incubation period, cells were pretreated with SDAPR, SDAP0, or SDAG for 24 h, and then exposed to cisplatin (25 µM, Civi Chemical Technology, China) or not for 24 h. After treatment, 20 µL MTT solution [15] (5 mg/mL, Biotopped, China) was added to the wells and incubated at 37°C for 4 h. Then, 150 µL DMSO (Shanghai Civi Chemical Technology, China) was added to each well, and the plates were agitated for 10 min. Finally, the absorbance was measured at 490 nm using a microplate reader (Nano, Germany).

Animals and experimental protocol

Animal experiments were designed and approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals. Animal experiments were executed by the Institutional Animal Ethic Committee of Department of Biochemistry, Jilin Agricultural University, Changchun, China.

ICR male mice (n = 40) weighing 20 ± 2 g were utilized in the studies, and were purchased from the Yisi Laboratory Animal Technology Co. Ltd. (SCXY-2011-0004). Mice were housed at controlled temperature ($28 \pm 2^\circ\text{C}$) and humidity ($65 \pm 5\%$) with 12 h light-dark cycle in animal cages. The experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice were freely fed rodent chow and tap water. Mice were divided into five groups of 8 animals each. Group I served as the control, and received distilled water (vehicle of SDAPR; p.o.) for 10 consecutive days and normal saline (vehicle of CDDP; i.p.) on the seventh day. Group II received distilled water for 10 consecutive days and CDDP (25 mg/kg; i.p.) dissolved with saline as a single intraperitoneal injection on the seventh day. Group III, IV, and V received a low-dose (5 mg/kg; p.o.), a middle-dose (10 mg/kg; p.o.), or a high-dose (20 mg/kg; p.o.), respectively of SDAPR solution for 10 consecutive days and CDDP (25 mg/kg; i.p.) dissolved with saline as a single intraperitoneal injection on the seventh day. On the tenth day, before mice were euthanized, blood samples were collected from ophthalmic veins. Renal tissues were collected immediately. One kidney was removed to determine histological analysis; the other was stored at -80°C for biochemical analysis.

Measurement of body weight and renal weight in mice

In the experimental period, the mice were weighed every day, and the rate of weight change was calculated. After the mice were sacrificed, the kidneys were quickly excised, cleaned, and weighed. Then, the kidney index was calculated as a ratio value of the renal weight over the body weight.

Determination of kidney function test

Blood urea nitrogen (BUN) and creatinine (Cr) levels were measured to assess renal function in mice. After the experimental period, blood samples were collected and centrifuged (3500 rpm, 15 min) to separate the serum. The levels of BUN and Cr were determined according to the commercial kit instructions (Nanjing Jiancheng Biotech. Co. Ltd.; Nanjing, China).

Measurement of oxidative stress markers

After the experimental period, oxidative stress markers were examined in mice, including super oxide dismutase (SOD), catalase (CAT), glutathione (GSH), and malondialdehyde (MDA). The activities of SOD, CAT, GSH, and the level of MDA were determined in renal tissues according to the commercial kit instructions (Nanjing Jiancheng Biotech. Co. Ltd.; Nanjing, China).

ELISA assay

After the experimental period, inflammation markers tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were examined in serum and renal tissues. The cytokine levels of TNF- α and IL-6 were detected in serum and renal tissues by mouse ELISA kits according to the manufacturer's instructions. All ELISA kits were purchased from Shanghai Lengton Bioscience Co., Ltd. (Shanghai, China).

Measurement of apoptosis markers

Western blot analysis was used to measure the total protein levels of cleaved-caspase-3, Bax, and Bcl-2 in renal tissues. Total proteins were extracted using RIPA (Beyotime, China). The BCA protein assay kit was used to quantify protein concentrations. Then, the protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were blocked with 5% skim milk powder overnight. Then, the membranes were incubated with the primary rabbit antibodies of caspase-3 (1:1000, Abcam, UK), Bax (1:500, Boster, China), Bcl-2 (1:500, Boster, China), and β -actin (1:500, Bioss, China) at 4°C overnight. Next, the membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies at room temperature for 2 h. Finally, the bands were visualized by ECL kit (Solarbio, Beijing, China) and analyzed by Image pro plus.

TUNEL assay

The terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay was used to measure the level of apoptosis in mice using the TUNEL Apoptosis Detection Kit (Nanjing Jiancheng Biotech, China). The numbers of apoptotic cell as defined by chromatin condensation or nuclear fragmentation were counted.

Histopathological evaluation

After the mice were euthanized, the right kidneys were collected and immediately embedded in OTC. Then, the sections were cut to 6 μ m pieces and stained with the H&E assay. Finally, renal histopathological sections were observed by light microscopy (Leica, Germany).

Statistical analysis

The results were expressed as the mean $\pm$ SD. One-way analysis of variance (ANOVA) was used to compare different groups. $P < 0.05$ and $P < 0.01$ were used to define the statistical significance.

Results

Effects of SDAPR, SDAPO, or SDAG treatment on CDDP-induced damage in HEK 293 cells

HEK 293 cells were treated with SDAPR, SDAPO, or SDAG before receiving CDDP. To determine the effects of these treatments on cell viability, the MTT method was used. As shown in Fig. 1, CDDP significantly reduced cell viability compared to the control group cells ($P < 0.01$), with a LD_{50} of approximately 25 μ M. Treatment with nine concentrations of SDAPR (0-2000 μ g/mL) for 24 h provided protection against CDDP-induced cell damage in a dose-dependent manner ($P < 0.05$), with an EC_{50} of approximately 1000 μ g/mL. However, the protection effect of SDAPO or SDAG was not significant.

Fig. 1. The effects of SDAPO, SDAG or SDAPR treatment on CDDP-induced damage in HEK293 cells.

Effect of SDAPR on PC-3M cell viability

In order to evaluate the toxicity of SDAPR on PC-3M cells, the cell viability was determined by MTT assay. As shown in Fig. 2A, the results indicate that cytotoxic levels of SDAPR affect PC-3M cells viability in a dose-dependent manner ($P < 0.05$). The LD₅₀ value of SDAPR is approximately 400 $\mu\text{g/mL}$. As shown in Fig. 2B, SDAPR (100-900 $\mu\text{g/mL}$) combined with CDDP (10 μM) with synergistic anti-tumor effect. These data demonstrated that the SDAPR treatment does not protect against tumor cells, and does not affect the therapeutic effect of CDDP.

Effect of SDAPR treatment on CDDP-induced kidney dysfunction in mice

Body weight and the kidney index were measured. As shown in Table 1, CDDP produced a significant weight reduction and kidney index increase compared with the control group. Compared with the mice that received CDDP alone, the mice that also received SDAPR showed significantly higher weights although still lower than the control group mice. Additionally, SDAPR pretreatment significantly decreased the kidney index ($P < 0.05$).

The levels of Cr and BUN were measured. As shown in Fig. 3, the levels of Cr and BUN were significantly increased after CDDP treatment, compared with the control group ($P < 0.01$). SDAPR pretreatment significantly decreased the levels of Cr and BUN compared to the levels in the group with only CDDP in a dose-dependent manner ($P < 0.05$).

Effect of SDAPR treatment on CDDP-induced oxidative stress

As shown in Fig. 4, the activities of antioxidant enzymes (SOD, CAT, and GSH) were significantly decreased, and the level of lipid peroxidation products (MDA) was significantly increased after CDDP administration *in vivo* ($P < 0.01$). Prior treatment with SDAPR markedly suppressed the MDA level and increased the activity of antioxidant enzymes compared to the CDDP only group ($P < 0.05$). These data indicate that SDAPR treatment provides partial protection to the CDDP-induced oxidative stress damage in mice kidneys.

Effect of SDAPR treatment on CDDP-induced inflammation

TNF- α and IL-6 levels are two key markers of inflammation. We evaluated the effect of SDAPR on CDDP-induced inflammation in serum and renal tissues by measuring the levels of TNF- α and IL-6 by ELISA. As shown in Fig. 5, the results showed that the secretions of TNF- α

Fig. 2. The effects of SDAPR treatment on cytotoxicity in PC-3M cells. (A) The PC-3M cells treated with SDAPR(0- 1000 $\mu\text{g/mL}$) for 24 h. (B) (A) The PC-3M cells treated with cisplatin (10 μM) and SDAPR(100- 1000 $\mu\text{g/mL}$) for 24 h.

Table 1. Effect of SDAPR treatment on CDDP-induced alterations in body weight and kidney index. Values are expressed as means $\pm$ SD (n = 6-8). * $p < 0.05$ vs. Control group. ** $p < 0.05$ vs. Control group. # $p < 0.05$ vs. CDDP group. ## $p < 0.01$ vs. CDDP group.

	Initial weight	Final weight	Change of weight	Mortality	Kidney index (%)
control group	27.22 $\pm$ 1.46	29.11 $\pm$ 2.28	2.68 $\pm$ 1.18	0/8	0.82 $\pm$ 0.10
CDDP group	31.08 $\pm$ 1.68	27.43 $\pm$ 1.67	-3.45 $\pm$ 1.51**	2/8	1.19 $\pm$ 0.06*
low-level group (5mg/kg)	28.78 $\pm$ 1.89	25.50 $\pm$ 0.78	-3.24 $\pm$ 1.68	1/8	0.886 $\pm$ 0.90*
middle-level group (10mg/kg)	30.06 $\pm$ 1.55	27.06 $\pm$ 0.86	-2.94 $\pm$ 1.83**	0/8	0.85 $\pm$ 0.06*
high-level group (20mg/kg)	29.50 $\pm$ 1.63	28.16 $\pm$ 0.71	-1.06 $\pm$ 1.63**	0/8	0.82 $\pm$ 0.12*

Fig. 3. Effect of SDAPR treatment on CDDP-induced alteration BUN and SCr levels in mice.

Fig. 4. Effect of SDAPR treatment on CDDP-induced the oxidative stress parameters in mice.

and IL-6 were dramatically increased after CDDP treatment ($P < 0.01$). However, SDAPR markedly reduced CDDP-induced inflammatory cytokine production ($P < 0.05$).

Effect of SDAPR treatment on CDDP-induced apoptosis

To evaluate the protective effect of SDAPR on CDDP-induced apoptosis, we analyzed the activities of caspase-3, Bax, and Bcl-2 in renal tissues. Apoptosis positive cells were identified by TUNEL staining. As the results in Fig. 6A show, SDAPR significantly decreased the CDDP-induced activity of Bax and increased activities of caspase-3 and Bcl-2 in a dose-dependent manner ($P < 0.01$). As shown in Fig. 6B, the renal apoptosis rate was significantly higher in the CDDP group compared to the control group ($P < 0.01$). Compared with the CDDP group, SDAPR significantly decreased the renal apoptosis rate ($P < 0.01$). These data suggest that SDAPR exerts inhibitory effects on CDDP-induced renal proximal tubule cell apoptosis.

Effect of SDAPR treatment on CDDP-induced kidney histopathological changes

As shown in Fig. 7, renal tissues showed a normal structure in the renal tubules (Fig. 7A, a). However, we observed diffuse acute tubular necrosis, cellular vacuolization, and

Fig. 5. Effects of SDAPR treated on CDDP-induced inflammatory parameters in serum and kidney tissues.

Fig. 6. Effect of SDAPR treated on CDDP-induced apoptosis in kidney injury. (A) Cleaved-caspase-3, Bax and Bcl-2 activities were determined by western blotting. (B) Effect of SDAPR on the apoptosis rate of kidney tissues.

cast formation in the CDDP group (Fig. 7B, b). Compared to the CDDP only group, SDAPR significantly attenuated the CDDP-induced histopathological changes in a dose-dependent manner (Fig. 7C-E, c-e).

Fig. 7. Histopathological changes of kidney tissues.

Discussion

CDDP-induced nephrotoxicity is a significant clinical problem. Hence, improved understanding of the pathophysiological processes behind CDDP-induced nephrotoxicity is critical for the development of new therapeutic strategies.

CDDP administration was previously found to affect renal function, and biochemical analysis showed that the levels of Cr and BUN renal function indicators were significantly increased after CDDP injection [16]. Our data show that SDAPR alleviated CDDP-induced renal function damage.

Previous studies demonstrated that CDDP alters kidney function and cause structural changes in kidneys. CDDP accumulates in renal tissues, damaging renal tubules and glomerulus [17]. The oxidative/nitrosative stress response, inflammation, and renal apoptosis are recognized as potential molecular mechanisms to explain the acute kidney injury caused by CDDP [4]. In this study, our results demonstrated that SDAPR treatment protected against CDDP-induced nephrotoxicity by limiting oxidative stress, inflammation response, and apoptosis.

Oxidative stress is a vital factor that leads to CDDP nephrotoxicity. Oxidative stress is an imbalance between oxidation and reduction processes, due to the generation of reactive oxygen species [18] that can induce lipid peroxidation production [19]. MDA was measured to assess the level of lipid peroxidation [20]. Previous work showed that CDDP administration results in the overproduction of free radicals. Antioxidant enzymes play critical roles in free radicals elimination [21]. For example, the decreased activity of GSH-px and SOD was significantly restored by morin [22] in CDDP treated mice, resulting in decreased free radical content. CDDP treatment causes a decrease in antioxidant enzyme activity in kidneys, likely from the loss of copper and zinc or by directly binding to sulfhydryl groups on cysteine. In this study, we examined antioxidant enzymes activity (GSH, SOD, and CAT) and lipid peroxidation products (MDA). Our results showed that SDAPR significantly enhanced antioxidant enzymes activity and decreased lipid peroxidation products.

Inflammation plays an important role in CDDP-induced nephrotoxicity *in vivo* and *in vitro*. Proinflammatory cytokines showed increased expression at the gene and protein levels after CDDP injection [23]. Among these inflammatory cytokines, TNF- α and IL-6 are major cytokines that lead to nephrotoxicity [24]. Previous work showed that CDDP administration elicited a 4-fold and 8-fold increase in TNF- α and IL-6, respectively [25]. Inflammatory cells are able to infiltrate renal tissues after CDDP injection [15]. The inhibition of inflammatory cytokines could attenuate renal tissue damage by CDDP [26]. Our results showed that SDAPR markedly reduced the levels of TNF- α and IL-6 in serum and kidneys.

Apoptosis is a common part of CDDP-induced nephrotoxicity. There is increased inflammation and oxidative stress within renal proximal tubule cells, causing apoptosis and necrosis. Reactive oxygen species play a crucial role in CDDP-induced apoptosis by mitochondrial pathway activation [27]. In addition, the receptor-mediated extrinsic pathway and the endoplasmic reticulum stress pathway are major pathways of apoptosis [28]. Caspase-3 is the most significant effector protease in apoptosis [29]. We evaluated caspase-3, Bax, and Bcl-2 protein expression using western blotting analysis. The results showed that SDAPR significantly ameliorated apoptosis protein expression.

Morphologically, CDDP nephrotoxicity is characterized by the loss of microvilli, tubular cell vacuolization, and tubular dilation [30]. In this study, we found that SDAPR obviously ameliorated CDDP-induced renal structure injury in mice.

In summary, these results suggest that SDAPR can be an effective dietary supplement to relieve CDDP-induced nephrotoxicity *in vivo*. The protective mechanisms may be attributed to improvement of antioxidant activity, inhibition of inflammation, and the suppression of renal tubular apoptosis by regulating caspase-3 expression.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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