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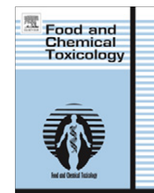

Carnosic acid attenuates renal injury in an experimental model of rat cisplatin-induced nephrotoxicity

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ABSTRACT

Nephrotoxicity is one of the serious dose limiting side effects of cisplatin when used in the treatment of various malignant conditions. Accumulating evidence suggests that oxidative stress caused by free radicals and apoptosis of renal cells contributes to the pathogenesis of cisplatin-induced nephrotoxicity. Present study was aimed to explore the effect of carnosic acid, a potent antioxidant, against cisplatin induced oxidative stress and nephrotoxicity in rats. A single dose of cisplatin (7.5 mg/kg) caused marked renal damage, characterized by a significant ($P < 0.05$) increase in serum creatinine, blood urea nitrogen (BUN) and relative weight of kidney with higher kidney MDA (malondialdehyde), tROS (total reactive oxygen species), caspase 3, GSH (reduced glutathione) levels and lowered tissue nitrite, SOD (superoxide dismutase), CAT (catalase), GSH-Px (glutathione peroxidase), GR (glutathione reductase) and GST (glutathione *S*-transferase) levels compared to normal control. Carnosic acid treatment significantly ($P < 0.05$) attenuated the increase in lipid peroxidation, caspase-3 and ROS generation and enhanced the levels of reduced glutathione, tissue nitrite level and activities of SOD, CAT, GSH-Px, GR and GST compared to cisplatin control. The present study demonstrates that carnosic acid has a protective effect on cisplatin induced experimental nephrotoxicity and is attributed to its potent antioxidant and antiapoptotic properties.

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1. Introduction

Cisplatin, an inorganic, divalent water-soluble and platinum-containing complex, is extensively used in the treatment of various malignant tumors of genital, urinary tract, head, neck, lung, adrenocortical carcinoma, etc. (Hanigan and Devarajan, 2003). However, the major clinical problem of cisplatin is its dose and duration-dependent nephrotoxicity, primarily affecting S3 segment of the proximal tubule (Jung et al., 2009). Despite the prophylactic measures like use of hydration, hypertonic saline, diuretics and other chemotherapeutic agents (Husain et al., 1998), approximately 25–35% of patients develop evidence of nephrotoxicity following a single dose of cisplatin due to its preferential accumulation within the proximal tubular epithelial cells (Kuhad et al., 2007; Fouad et al., 2008).

There is substantial evidence suggesting the role of nitric oxide (Kuhad et al., 2007; Saleh and El-Demerdash, 2005; Saad et al., 2000; Yildirim et al., 2003), oxidative stress and lipid peroxidation caused by oxygen-centered free radicals generation (Ajith et al., 2007; Atessahin et al., 2005; NazIroglu et al., 2004; Matsushima et al., 1998) and apoptosis (Santos et al., 2007, 2008) associated with the pathogenesis of cisplatin-induced nephrotoxicity. Apart from this, multiple lines of recent evidence suggest that the role of inflammatory mechanisms through the recruitment of inflammatory cells, such as macrophages and leukocytes and generation of inflammatory cytokines like TNF-α (Ramesh and Reeves, 2002; Sung et al., 2008), mitochondrial dysfunction (Rodrigues et al., 2009), up regulation of mitochondrial nitric oxide synthase and peroxynitrite formation (Jung et al., 2009), increased up-regulation of the adenosine A1 receptor in kidney (Bhat et al., 2002), generation of toxic metabolite cisplatin–glutathione-conjugate

Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; BUN, blood urea nitrogen; BW, body weight; CA, carnosic acid; CAT, catalase; DCFH-DA, 2,7-dichlorofluorescein diacetate; DTNB, 5,5-dithio-bis (2-nitrobenzoic acid); NED, *N*-(1-naphthyl)-ethylene-diamine dihydrochloride; GR, glutathione reductase; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSSH, glutathione oxidized; GST, glutathione *S*-transferase; i.p., intra peritoneal; MDA, malondialdehyde; NADPH, β-nicotinamide adenine dinucleotide 3-phosphate; NO, nitric oxide; PAS, periodic acid schiff; SOD, superoxide dismutase; tROS, total reactive oxygen species.

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(Townsend et al., 2003), etc. playing key role in the development of cisplatin induced nephrotoxicity.

Rosemary, a well known aromatic plant, has been used in the food industry to flavour and to prevent oxidation of meat or stews. Rosemary oil finds use in the production of bath products, detergents and insecticides. The published data on the antioxidant effect of rosemary extracts is attributed to the phenolic diterpenes such as carnosol, carnosic acid, methyl carnosate and phenolic acids such as rosmarinic and caffeic acids. Carnosic acid, a naturally occurring catechol type poly-phenolic diterpene present in rosemary (*Rosmarinus officinalis*; Lamiaceae) has a wide array of pharmacological and biological activities such as antioxidant (Aruoma et al., 1992, 1996; Kuzmenko et al., 1999; Romano et al., 2009; Posadas et al., 2009), antimicrobial (Romano et al., 2009), neuroprotective (Satoh et al., 2008a,b; Park et al., 2008), hepatoprotective (Sotelo-Felix et al., 2002), antiobesity (Ninomiya et al., 2004), anti-inflammatory (Poeckel et al., 2008), anticancer (Shabtay et al., 2008) and antidepressant properties (Machado et al., 2009), etc. It is also reported that carnosic acid, a lipophilic antioxidant, prevents lipid peroxidation and disruption of biological membranes by scavenging singlet oxygen, hydroxyl radicals and lipid peroxyl radicals (Zhang et al., 2010). Therefore, it seems reasonable to investigate whether administration of carnosic acid protects nephrotoxicity or not and also to examine its antioxidant and antiapoptotic properties against cisplatin-induced nephrotoxicity.

2. Materials and methods

2.1. Drugs and chemicals

Cisplatin, reduced glutathione (GSH), glutathione oxidized (GSSG), NADPH (β-nicotinamide adenine dinucleotide 3-phosphate), 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB), glutathione reductase, 2-thiobarbituric acid (TBA), glutathione peroxidase, catalase, 2,7-dichlorofluorescein diacetate (DCFH-DA), caspase 3 assay kit, fluorimetric, superoxide dismutase assay kit were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Avra synthesis Pvt. Ltd., Hyderabad, India. Hydrogen peroxide was purchased from Fisher Scientific, Mumbai, India. Sulphanilamide was obtained from Loba Chemie Pvt. Ltd., Mumbai and *N*-(1-naphthyl)-ethylene-diamine dihydrochloride (NED) were from S.D. Fine chem limited, Mumbai, India. Carnosic acid (60%) from rosemary plant was kind gift from Exotic Naturals Pvt. Ltd., Mumbai, India. All other chemicals were of analytical grade and were obtained commercially.

2.2. Animals

Female Wistar rats weighing between 170 and 190 g were obtained from National Institute of Nutrition (NIN), Hyderabad, India. The animals were housed in a temperature-controlled environment with a 12-h light/dark cycle with controlled temperature (24 ± 3 °C and relative humidity 55 ± 15%) during the experimental period and were allowed free access to food and water at all times. The protocol of this study was approved by the Institutional Animal Ethical Committee (IAEC) of Indian Institute of Chemical Technology (IICT), Hyderabad, India (IICT/PHARM/SRK/01/01/10). Experimental procedures were conducted in accordance with the CPCSEA guidelines on the safe use and care of experimental animals.

2.3. Experimental design

The animals were randomly divided into five groups each containing six rats. Cisplatin was injected intraperioneally at the dose of 7.5 mg/kg BW for induction of nephrotoxicity in rats based on the pilot studies. The experimental design was given below. All the animals were sacrificed on 6th day of cisplatin administration.

(1) *Normal control: single* intra peritoneal (i.p.) injection of 0.5 ml isotonic saline was given and 2% gum acacia suspension was administered per orally for 10 days.
(2) *CA control: rats* were given a suspension of carnosic acid (100 mg/kg BW/day) 2% in gum acacia per oral for 10 days.
(3) *Cis control: single* intraperitoneal (i.p.) injection of cisplatin (7.5 mg/kg BW, i.p.) dissolved in normal saline was given.
(4) *CA (100 mg/kg) (pre) + cis:* rats received a single dose cisplatin (7.5 mg/kg BW, i.p.) following 5 days of carnosic acid (100 mg/kg p.o.) administration, pre-treatment.
(5) *CA (100 mg/kg) (pre and post) + cis:* rats were administered carnosic acid (100 mg/kg BW/day) orally for 10 consecutive days in addition to cisplatin (7.5 mg/kg, i.p.) which was administered as a single intraperitoneal dose on the 5th day of the experiment 1 h prior to CA dose.

2.4. Sampling and biochemical assays

On 6th day (i.e. on 6th day) of cisplatin administration, body weights of rats were recorded and blood samples, a mixture of arterial and venous blood were collected from intra cardiac puncture and then euthanized in CO_2 chamber. Kidney was dissected, weighed and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Kidney to body weight ratio was calculated. Kidney was minced into small pieces and homogenized in ice-cold phosphate buffer saline (PBS) (0.05 M, pH 7) to obtain 1:9 (w/v) whole homogenate. A part of the homogenate was taken and mixed with equal volume of 10% trichloroacetic acid (TCA) and centrifuged at 5000 rpm for 10 min and supernatant was used for the estimation of GSH and MDA. The remaining part was centrifuged at 17,000*g* for 60 min at 4 °C, and supernatants was used for the assay of protein, CAT, GSH-Px, SOD, GR, GST, ROS and tissue nitrite levels. The kidney samples of rats from different experimental groups were fixed in 10% neutral buffered formalin for 24 h.

2.4.1. Assessment of renal functions

2.4.1.1. Estimation of blood urea nitrogen and serum creatinine levels. Blood urea nitrogen (BUN) and creatinine in serum were determined using BUN *AUTOPAK* kit and CRTN *AUTOPAK* kit (Siemens, Baroda, India), respectively, using auto blood analyzer (Bayer Corporation, USA).

2.4.2. Assessment of nitrosative stress by estimation of renal nitrite levels

The nitric oxide (NO) production was measured by estimating the accumulation of nitrites (NO^{2-}) in the supernatant (Gonzalez-Barrios et al., 2002) with slight modification by Griess reagent system (Promega technical bulletin, USA). Briefly, to a volume of 50 μl of supernatant, sulfanilamide (1% sulfanilamide in 5% phosphoric acid, 50 μl) solution was added and incubated for 5–10 min at room temperature, protected from light. Then a volume of 50 μl of the NED (0.1% *N*-1-napthylethylenediamine dihydrochloride in water) solution was added and allowed to incubate for 5–10 min at room temperature, protected from light. A purple/magenta color, thus formed, was measured at 540 nm using micro plate reader (Biotek instruments, Synergy 4, USA) and interpolated to a standard curve of $NaNO_2$ (0–100 μM) to calculate the nitrite content. The nitrite levels were expressed as μmol/g wet tissue.

2.4.3. Assessment of oxidative stress

2.4.3.1. Estimation of renal total reactive oxygen species (ROS). Reactive oxygen species (ROS) levels in the kidney tissue were determined fluorometrically by using 2,7-dichlorofluorescein diacetate (DCF-DA) (Maiti et al., 2010). Briefly, to 10 μl of 100 μM DCF-DA (dissolved in DMSO), a volume of 90 μl of supernatant was added and incubated for 30 min at room temperature in the dark. After incubation, the volume was made up to 3 ml using PBS (0.1 M, pH 7.4) and the fluorescence was measured at an excitation wavelength of 488 nm and emission wavelength of 525 nm using micro plate reader (Biotek Instruments, synergy 4, USA). The result was expressed as percentage change fluorescence, where the normoxia group was taken as 100%.

2.4.3.2. Estimation of renal lipid peroxidation (MDA). The concentration of MDA in kidney homogenate as an index of lipid peroxidation was determined based on the reaction with thiobarbituric acid (Ohkawa et al., 1979). MDA levels were quantified using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nanomoles of MDA per g of tissue.

2.4.3.3. Estimation of reduced glutathione (GSH). Tissue GSH concentration was measured by the method as described by Ellman (Ellman, 1959) and was expressed as μmol/g tissue.

2.4.3.4. Estimation of catalase (CAT). Catalase activity in kidney tissue was determined by measuring the rate of decomposition of hydrogen peroxide at 240 nm (Aebi, 1974) and the activity was expressed as U/mg protein.

2.4.3.5. Estimation of superoxide dismutase (SOD). Total SOD activity (cytosolic and mitochondrial) was determined using SOD assay kit (Sigma–Aldrich Co., St. Louis, MO, USA) according to the manufacturer specifications and was expressed as percentage activity while the normoxia group was taken as 100%.

2.4.3.6. Estimation of glutathione peroxidase (GSH-Px). Glutathione peroxidase activity was determined spectrophotometrically (Paglia and Valentine, 1967) with slight modification by Wandel (1981). Briefly, to a mixture containing 500 μl of potassium phosphate buffer containing EDTA and sodium azide (0.25 M, pH 7), volumes of 100 μl each of glutathione reductase (30 U), GSH (10 mM), NAPDH (2.5 mM) and supernatant and H_2O_2 (1 mM) were added, mixed and changes in absorbance were

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