

Biochemical Studies on Nephroprotective Effect of Carob (*Ceratonia siliqua* L.) Growing in Egypt

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ABSTRACT: Reactive oxygen species and free radicals are involved in the nephrotoxicity induced by the synthetic anticancer drug cisplatin. The nephroprotective effect of carob pods and leaves (100 and 200 mg/kg, p.o.) was investigated using cisplatin (10 mg/kg body weight, i.p.) to induce oxidative renal damage in mice. The results showed that cisplatin administration caused abnormal renal functions in all studied mice. Serum urea and creatinine concentrations were significantly highered ($P<0.5$) in the cisplatin alone treated (control) group compared to the normal group. The concentrations of serum creatinine and urea in the carob pods (200 mg/kg body weight) treated group were reduced to 57.5% and 51.5%, respectively, with respect to the control group. Also, cisplatin induced decline of renal antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities, but the treatment of carob pods and leaves (100 and 200 mg/kg, p.o.) significantly attenuated the cisplatin-induced nephrotoxicity. Both pods and leaves of carob at 100 and 200 mg/kg increased the concentration of reduced glutathione (GSH) and protected against the increase of cisplatin-induced lipid peroxidation. In addition, treatment with cisplatin increased the activity of cathepsin D, RNase II, DNase II and acid phosphatase. The treatment of carob pods and leaves (100 and 200 mg/kg, p.o.) improved the activity of lysosomal enzymes nearly to the normal group. In conclusion, carob pods and leaves may be effective to protect from oxidative renal damage and the leaves are the better nephroprotective agent than pods. The protection may be mediated partially by preventing the decline of renal antioxidant statuses. [Nature and Science 2010;8(3):41-47]. (ISSN: 1545-0740).

Key words: nephrotoxicity; carob; cisplatin; antioxidant enzymes; lysosomal enzymes

INTRODUCTION

Cisplatin is a widely used antineoplastic agent for the treatment of metastatic tumors of the testis, metastatic ovarian tumors, lung cancer, advanced bladder cancer and many other solid tumors (Sweetman, 2002). The cytotoxic action of the drug is often through its ability to bind DNA to form cisplatin-DNA adducts (Goldstein and Mayor, 1983). Although higher doses of cisplatin are more efficacious for the suppression of cancer, high dose therapy manifests irreversible renal dysfunction and other toxicities yet (Simic and Jovanovic, 1986 and Halliwell and Cross, 1994). Various data indicate that cisplatin induces oxidative stress (Ajith *et al.*, 2002), lipid peroxidation (Bompert, 1989 and Matsushima *et al.*, 1998) and DNA damage (Liberthal *et al.*, 1996). Therefore administration of antioxidants has been shown to ameliorate cisplatin-induced nephrotoxicity in various species of animals (Somani *et al.*, 2000). The mechanism of protective effects of antioxidants against cisplatin nephrotoxicity is not fully known.

Ceratonia siliqua L., Fabaceae (Carob) has been widely cultivated in Mediterranean countries for years. This tree was distributed by Arabs in the Mediterranean area (Kumazawa *et al.*, 2002). The plant is grown locally in Egypt, and the pods are used mainly for preparing a popular beverage. Leaves and pods of carob exerted diverse physiological function as antioxidant activity (Kumazawa *et al.*, 2002 and Rasheed, 2006).

Custódio *et al.* (2009) investigated the Antioxidant activity and *in vitro* inhibition of tumor cell growth of carob tree (*Ceratonia siliqua*). The methanol extracts of the carob tree showed significant radical scavenging activity and a remarkable ability to inhibit tumor cell proliferation. Carob pods and leaves extracts contain antiproliferative agents that could be practical importance in the development of functional foods and/or chemopreventive drugs. In addition leaves and pods of carob are rich in polyphenols and flavonoids (Rasheed, 2006).

In the present study, the protective effects of carob pods and leaves by two doses (100 and 200 mg/kg, p.o.) on cisplatin-induced renal damage in mice were evaluated.

MATERIALS AND METHODS

Preparation of samples

Carob (*C. Siliqua*) pods and leaves samples were obtained from Experimental Station of Medicinal Plants, Giza. The pods and leaves were grinded to fine powder before extraction. Such powdered samples were kept in dark bottles.

Chemicals

Cisplatin (1mg/ml) Onco-Tain DBL was from Mayne Pharma PLC, UK., Reduced glutathione (GSH), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), EDTA and thiobarbituric acid (TBA) were from Sigma-Aldrich Co, St Louis,

USA. All other chemicals and reagents used were of analytical grade.

Animals

Albino male mice (30±6 g) were used in the present study. The mice were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR), Egypt. The animals were kept under standard laboratory conditions of light/dark cycle (12/12h) and temperature (25±2°C). They were provided with a nutritionally adequate standard laboratory diet.

Animals' diet

The basal diet consists of casein 10%, cotton seed oil 4%, salt mixture 4%, vitamin mixture 1%, carbohydrates (sucrose, starch 1:1) 80.8% and choline choride 0.2% (**American Institue of Nutrition, 1980**).

Plant extracts

One hundred gram of pods and leaves of carob were separately extracted by percolation with 70% ethanol. The extracts were filtered, concentrated under vacuum and freeze dried.

Experimental design

Animals were divided into 6 groups of 6 animals each.

Group I treated with vehicle (gum acacia, 1%) was kept as normal.

Group II injected with a single dose of cisplatin (CIS) (10 mg/kg b.wt., i.p.) was kept as control

Group III and IV were treated with pods extract (P.), 100 and 200 mg/kg b.wt.

Group V and VI were treated with leaves extract (L.), 100 and 200 mg/kg b.wt.

The pods and leaves extracts were freshly prepared as fine suspension in gum acacia and administered by oral gavage one h before and 24 h and 48 h after cisplatin injection. Seventy two hours after cisplatin injection, animal were killed by cervical decapitation. Blood was collected and the separated serum was used for the estimation of creatinine (**Bartless et al., 1972**) and urea (**Patton and Crouch, 1977**). After decapitation, kidney was rapidly removed and washed in cold isotonic saline. The kidney was divided into two portions. The first one was homogenized in 50 mM phosphate buffer (pH 7) using an electronic homogenizer to prepare

10 % w/v homogenate. The homogenate was centrifuged at 3000 rpm for 10 min. at 4°C and the supernatant was used for the estimation of total protein (**Lowry et al., 1951**), lipid peroxidation (TBARS) measured as malondialdehyde (MDA) (**Ohkawa et al., 1979**), superoxide dismutase (SOD) (**Marklund and Marklund, 1974**), catalase (CAT) (**Takahara et al., 1960**), Glutathione peroxidase (GPx) (**Rotruck et al., 1973**), reduced glutathione (GSH) (**Ellman, 1959**) and glutathione-S-transferase (GST) (**Habig et al., 1974**). The second portion was used for lysosomal isolation according to **Harikumar et al., (1989)**. The activities of four lysosomal acid hydrolases were measured. Cathepsin D, RNase II, DNase II and acid phosphatase activities were determined according to the method of **Gianetto and de Duve (1955)** and **Barett and Heath (1977)**.

Statistical analysis

The results are expressed as Mean±SEM. The collected data were statistically analysed by the least significant differences (LSD) at the level 5% of the probability procedure according to **Snedecor and Cochran (1980)**.

RESULTS

Intravenous cisplatin administration caused abnormal renal functions in all injected mice. Serum urea and creatinine concentrations were significantly increased (P<0.5) in the cisplatin alone treated (control) group compared to the normal group (Table 1). The concentrations of serum creatinine and urea in the carob pods (200 mg/kg body weight) treated group were reduced to 57.5% and 51.5%, respectively, with respect to the control group. Similarly, the concentrations of urea and creatinine in the carob leave (200 mg/kg) treated group were reduced to 62.8% and 65.2%, respectively.

The activities of renal SOD, CAT and GPx in the cisplatin plus carob pods or cisplatin plus carob leaves administered group are given in Table 2. Renal SOD activity was decreased significantly (P<0.05) in the cisplatin alone treated group compared to the normal group. The SOD activity in the carob pods and leaves (200 mg/kg body weight) administered groups were increased significantly (P<0.05) when compared to that of control group.

Table (1): Effect of carob pods and leaves on serum urea and creatinine in mice treated with cisplatin (CIS).

Groups	Urea (mmol/L)	Creatinine (mmol/L)
	Mean±SE	Mean±SE
Normal	6.8±1.1	28.1±4.2
Control (CIS)	23.1±2.1 ^a	260.4±50.2 ^a
P100+ CIS	15.7±1.0 ^b	152.6±13.6 ^b
P200+ CIS	11.2±1.2 ^b	110.7±27.9 ^b
L100+ CIS	14.2±1.4 ^b	108.2±10.9 ^b
L200+ CIS	8.6±1.0 ^b	90.5±12.2 ^b

Values are Mean±SEM (n=6 animals). ^a p<0.05, (Student's *t test*) significantly different from normal group. ^b p<0.05, significantly different from control group. P, pods and L, leaves of carob.

Table (2): Effect of carob pods and leaves on renal SOD, CAT and GPx in mice treated with cisplatin (CIS).

Groups	SOD (nmol/min/mg protein)	CAT (nmol/min/mg protein)	GPx (nmol/min/mg protei)
	Mean±SE	Mean±SE	Mean±SE
Normal	22.2±2.6	160.9±6.8	253.1±6.1
Control (CIS)	10.6±1.9 ^a	87.7±2.8 ^a	130.7±3.5 ^a
P100+ CIS	15.2±2.8 ^{ns}	139.1±2.6 ^b	157.2±3.0 ^{ns}
P200+ CIS	17.2±3.1 ^b	156.9±2.8 ^b	222.9±4.1 ^b
L100+ CIS	17.9±2.7 ^b	1452.8±3.3 ^b	220.1±3.2 ^b
L200+ CIS	19.1±3.6 ^b	158.1±5.2 ^b	248.2±5.2 ^b

Values are Mean±SEM (n=6 animals). ^a p<0.05, (Student's *t test*) significantly different from normal group. ^b p<0.05, significantly different from control group. ns, non significant different from control group. P, pods and L, leaves of carob.

The activity of CAT in the cisplatin alone treated group was found to be decreased significantly (P<0.05) when compared to the normal group. Treatment of carob pods and leaves effectivity prevented the cisplatin induced decline of the CAT activity. Similarly, GPx activity was decreased significantly in cisplatin treated group. The enzyme activity was significantly increased (P<0.05) except at low dose of carob pods that could not prevent the decline of GPx activity.

The concentration of renal GSH was significantly decreased (P<0.05) and that of malondialdehyde was significantly increased (Table 3) in cisplatin treated animals. Administration of carob pods or leaves prior to cisplatin injection increased GSH and decreased the MDA concentrations.

Administration of cisplatin induced significant decrease in renal GST activity in comparison to normal value Table (3), whereas, carob pods and leaves (200 mg/kg) significantly ameliorated the effect of cisplatin compared to cisplatin group.

The effects of cisplatin treatment on lysosomal enzyme activities are presented in Table 4. Cisplatin treatment increased the activities of the four lysosomal enzymes; cathepsin D, acid phosphatase, DNase II and RNase II, significantly (P<0.05) compared to normal group. Administration of carob pods or leaves by two doses prior to cisplatin injection significantly (P<0.05) ameliorated the effect of cisplatin in all enzyme activities, compared to control group.

Table (3): Effect of carob pods and leaves on renal GSH, TBARS and GST in mice treated with cisplatin (CIS).

Groups	GSH (n mol/ mg protein)	TBARS (n mol/mg protein)	GST (nmol/min/ mg protein)
	Mean±SE	Mean±SE	Mean±SE
Normal	5.0±0.6	1.5±0.20	20.1±3.9
Control (CIS)	2.4±0.5 ^a	3.6±0.26 ^a	11.9±1.2 ^a
P100+ CIS	3.8±1.0 ^{ns}	3.1±0.16 ^{ns}	15.1±4.0 ^{ns}
P200+ CIS	4.2±0.7 ^b	1.8±0.19 ^b	18.9±3.5 ^b
L100+ CIS	3.9±0.2 ^b	1.9±0.15 ^b	17.1±5.1 ^b
L200+ CIS	5.1±1.1 ^b	1.6±0.21 ^b	19.0±3.0 ^b

Values are Mean±SEM (n=6 animals). ^a p<0.05, (Student's *t test*) significantly different from normal group. ^b p<0.05, significantly different from control group. ns, non significant different from control group. P, pods and L, leaves of carob.

DISCUSSION

Cisplatin has been shown to cause nephrotoxicity in patients (De Conti *et al.*, 1973 and Daugarrd *et al.*, 1988) as well as in a variety of animal species (Mckeage *et al.*, 1993 and Badary *et al.*, 1997 a,b). A minimum dose of cisplatin (5 mg/kg b.wt, i.p.) was sufficient to induce nephrotoxicity in rats (Boogaard *et al.*, 1991 and Ravi *et al.*, 1995). A higher dose of cisplatin (10 mg/kg b.wt. i.p) corresponds to that currently being used in clinical practice.

Administration of cisplatin exerts significant increase in serum urea and creatinine concentrations compared to normal group, which clearly indicates the acute renal failure. The effect of cisplatin was similar to those previously described (Heidemann *et al.*, 1989; Mckeage *et al.*, 1993 and Somani *et al.*, 2000). Carob pods and leaves ameliorated cisplatin-induced nephrotoxicity as indicated by significant less increase in serum urea and creatinine concentrations.

Table (4): Effect of carob pods and leaves on renal Cathepsin D, Acid Phosphatase, DNase II and RNase II in mice treated with cisplatin (CIS).

Groups	Cathepsin D (nmol/min/mg protein)	Acid phosphatase (nmol/min/ mg protein)	DNase II (nmol/min/ mg protein)	RNase II (nmol/min/ mg protein)
Normal				
Mean±SE	30.0±9.5	45±0.02	0.10±0.02	0.30±0.04
Control (CIS)				
Mean±SE	68.2±10.1 ^a	97±0.09 ^a	0.52±0.01 ^a	0.81±0.09 ^a
P100+ CIS				
Mean±SE	35.2±5.9 ^b	52±0.09 ^b	0.26±0.07 ^b	0.62±0.05 ^b
P200+ CIS				
Mean±SE	34.1±6.8 ^b	48±0.07 ^b	0.21±0.05 ^b	0.48±0.06 ^b
L100+ CIS				
Mean±SE	31.2±9.7 ^b	51±0.09 ^b	0.20±0.08 ^b	0.53±0.08 ^b
L200+ CIS				
Mean±SE	29.2±8.9 ^b	48±0.06 ^b	0.18±0.06 ^b	0.44±0.07 ^b

Values are Mean±SEM (n=6 animals). ^a p<0.05, (Student's *t test*) significantly different from normal group. ^b p<0.05, significantly different from control group. P, pods and L, leaves of carob.

The renal antioxidant status, such as SOD, CAT, GPx activities and GSH concentration are significantly decreased in the cisplatin alone treated group of animals compared to normal group. The decline of antioxidant status partially explains the mechanism of nephrotoxicity induced by cisplatin. The renal accumulation of platinum and covalent binding of platinum to renal protein could, also, play a role in the nephrotoxicity (Litterst and Schweitzer, 1988). Cisplatin induced suppression of renal antioxidant enzyme activities was also supported by the published experimental results (Ajith *et al.*, 2002 and Cetin *et al.*, 2006).

Carob pods and leaves (200 mg/kg b.wt. i.p.) along with cisplatin could significantly improve the depletion of the renal antioxidant system.

GSH depletion increases the sensitivity of organ to oxidative and chemical injury. Studies with a number of models show that the metabolism of xenobiotics often produced GSH depletion (Mitchell *et al.*, 1973, Jollow *et al.*, 1974 and Ahmed and Zaki, 2009). The depletion of GSH, also, seems to be a prime factor that permits lipid peroxidation in the cisplatin treated group. Treatment of carob pods and leaves reduced the depletion of GSH levels and provided protection to the kidney. The protection of GSH is by forming the substrate for GPx activity that can react directly with various aldehydes produced from the peroxidation of membrane lipid.

The initiation and propagation of lipid peroxidation in the cisplatin treated group could be caused by the decreased SOD activity. Such

decreased activity may be either due to loss of copper and zinc, which are essential for the activity of enzyme or due to reactive oxygen species-induced inactivation of the enzyme protein (Sharma, 1985 and De Woskin and Riviere, 1992).

The activity of CAT and GPx, also, decreased in the cisplatin treated group, which in turn increased the hydrogen peroxide concentration and enhanced the lipid peroxidation. Hence the concentration of MDA, as a result of lipid peroxidation, increased in the cisplatin treated group. Treatment with leaves and pods of carob prevented the lipid peroxidation by enhancing the renal SOD, CAT and GPx activities. It is well known that many phenolic compounds, which are found in carob, exert powerful antioxidant effects. They, also, inhibit lipid peroxidation by scavenging reactive oxygen species (ROS), such as OH⁰ (Shahidi and Wanasundara, 1992).

From the data presented (Table 4), it is clear that cisplatin treatment in general resulted in increase in the activity of all lysosomal enzymes under study. In the carob pods and leaves treated groups this effect was improved nearly to the normal group.

There is a correlation between lipid peroxidation and the release of lysosomal enzymes from lysosomes. Hence the process of lipid peroxidation activates phospholipases and removes the peroxidized lipid from the membrane (Kappus, 1985). The oxidation of unsaturated fatty acids in biological membranes by free radical leads to a decrease in membrane fluidity and disruption of membrane structure and function (Haragushi *et al.*, 1997).

The increase in activities of RNase II and DNase II is a matter of concern, since this can lead to indiscriminate degradation of RNA and DNA ultimately resulting in necrosis of the cells in the tissues, i. e. kidney and liver (Patel *et al.*, 2005). Also, Gianetto and de Duve (1955) reported that the cathepsin D activity increased substantially in experimental thyrotoxicosis. It was found that the acid phosphatase activity increased after cisplatin treatment (Table 4). Panya *et al.* (2003) showed that lysosomal acid phosphatase preferentially acts on nucleotides and that AMP is the preferred substrate. The concerned action of activated nucleases and acid phosphatase would lead not only to the breakdown of nucleic acids but also to the further dephosphorylation of mononucleotides, thereby leading to the acceleration of the process of cell degeneration.

Ethanollic extract of carob leaves possessed strong radical scavenging activity *in vitro* as measured by DPPH assay. Furthermore, the *in vivo* studies confirmed the antioxidant efficacy of this extract as well as its hepatoprotective activity (Rasheed, 2006). Polyphenols in carob pods have

antioxidant activity (Kumazawa *et al.*, 2002). In addition the crude polyphenol extracts of carob pods showed strong antioxidant activity (Harbore, and Baxter, 1999). The protective effect of carob pods and leaves, in the present study, against cisplatin-induced nephrotoxicity is in harmony and supports the previous reports indicating the antioxidant and cytoprotective potential of carob pods and leaves.

In conclusion, carob pods and leaves improve the nephrotoxicity of cisplatin in mice. The nephroprotective effects of carob pods and leaves may be partially mediated by preventing the cisplatin-induced decline of renal antioxidant status and lysosomal membrane damage.

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