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In Vitro and *In Vivo* Effects of Phenolic Antioxidants against Cisplatin-Induced Nephrotoxicity

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We have investigated the effect of phenolic antioxidants on cisplatin-induced cytotoxicity in vero (African Green Monkey Kidney) cells and in rat renal cortical slices *in vitro*, and on cisplatin-induced nephrotoxicity in rats *in vivo*. Incubation of cisplatin with vero cells resulted in time- and concentration-dependent cytotoxicity, as characterized by decreased tryphan blue exclusion (TBE) and increased release of lactate dehydrogenase (LDH) into the medium. Cisplatin also caused reduction of glutathione (GSH) in a concentration-dependent manner. In the rat renal cortical slices model, incubation of cisplatin for 120 min caused an increase in malondialdehyde (MDA), a decrease in GSH and inhibited *p*-aminohippurate (PAH) uptake in a concentration-dependent manner. Among phenolic antioxidants, isoeugenol (IG) was found to be more active against cisplatin-induced cytotoxicity in vero cells as well as in rat renal cortical slices than eugenol (EG) and dehydrozingerone (DZ). However none of the test compounds were able to arrest the reduction of the GSH content induced by cisplatin in either the vero cells or the renal cortical slice model. Administration of cisplatin (3 mg/kg) i.p. to rats resulted in significant reduction of body weight, and elevation of blood urea nitrogen (BUN) and serum creatinine. Treatment with IG 10 mg/kg i.p. 1 h before cisplatin resulted in partial but significant protection against the cisplatin-induced reduction of body weight, and elevation of BUN and serum creatinine, the protection being 34, 46, and 62%, respectively. EG and DZ (10 mg/kg, i.p.) were found to be inactive *in vivo*. Because IG is a potent free radical scavenger and protects against cisplatin-induced toxicitiy, the present results have many clinical implications in cisplatin chemotherapy and thus warrants further investigation.

Key words: cisplatin, cytotoxicity, free radicals, nephrotoxicity, phenolic antioxidants.

Cisplatin is an important antineoplastic agent used for several types of solid tumors (*1*). Its clinical use is limited by its renal toxicity (*2, 3*). Although the mechanism of action of the cisplatin renal toxicity is still not clear, it has been suggested that oxygen free radicals play an important role (*4-11*). Cisplatin is known to cause increased lipid peroxidation and inhibition of *p*-aminohippurate (PAH) uptake in renal cortical slices (*11*). Many antioxidants are known to partially protect the kidney from the cisplatin toxicity (*4, 6, 12-14*). It has been reported that a crude extract of *Gingko biloba*, a well known antioxidant, protected renal cortical slices against cisplatin-induced and inhibition of PAH uptake (*11*). *O*-(β-Hydroxyethyl)-rutoside, a bioflavanoid antioxidant, prevents cisplatin-induced acute renal failure in rats (*14*). Cystone extract, a polyayurvedic preparation used clinically for many urinary complications, has been shown to provide partial but significant protection against renal toxicity induced by cisplatin (*10*).

IG and EG are phenolic compounds present in the volatile oils of several plants (*15*). Both IG and EG are good inhibitors of lipid peroxidation, and act as potent free radical scavengers (*16, 17*). DZ is a structurally related compound and known to be a metabolite of curcumin, a well-known antiinflammatory agent (*18-20*). Like IG and EG, DZ is also a good inhibitor of lipid peroxidation and acts as a good free radical scavenger (*17, 21, 22*).

The purpose of the present study was to evaluate the effects of IG, EG, and DZ against cisplatin-induced toxicitiy *in vitro* and *in vivo* since they are potent antiperoxidative agents.

MATERIALS AND METHODS

Materials—Cisplatin, eugenol (EG), *p*-aminohippuric acid (PAH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), tryphan blue, 2,2′-naphthylethylenediamine hydrochlo-

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Abbreviations: BUN, blood urea nitrogen; DZ, dehydrozingerone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EG, eugenol; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; IG, isoeugenol; LDH, lactate dehydrogenase; MDA, malondialdehyde; NADH, nicotinamide adenine dinucleotide (reduced form); PAH, *p*-aminohippurate; TBA, thiobarbituric acid; TBE, tryphan blue exclusion.

ride, thiobarbituric acid (TBA), and trypsin were purchased from Sigma, St. Louis, MO, USA. Isoeugenol (IG) was obtained from Industrial Perfumes, Mumbai, India. Dehydrozingerone (DZ) was synthesized in our laboratory (*19*). All other chemicals were of high purity and obtained from S.D. Fine Chemicals or Merck (India). All the chemicals for cell culture experiments were obtained from Himedia, Mumbai.

Drug Solutions—Cisplatin was dissolved in distilled water to give a 1 mg/ml stock solution. IG, EG, or DZ was dissolved in 0.05 N NaOH, and the pH was adjusted to 7.4 with 0.1 N HCl for *in vitro* experiments. For *in vivo* studies IG, EG, or DZ was suspended in 1% Tween 80 to give a 1 mg/ml stock solution.

Cell Culture—Vero (African Green Monkey Kidney) cells were originally procured from the National Cell Culture Facility, Pune, India. The cells were maintained in Dulbecco's Minimum Essential Medium (Catalog no. AT007; Himedia, Mumbai) supplemented with amino acids (Catalog no. AC004; Himedia, Mumbai) and polyethylene glycol 6000-treated (to separate immunoglobulins, particularly δ-globulins) 5% newborn calf serum (obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore, India) without antibiotics, under a humidified atmosphere of 95% air-5% carbon dioxide at 37°C. Confluent monolayers were subcultured using 0.05% trypsin-0.53 mM EDTA in Ca^{2+}-Mg^{2+} free phosphate-buffered saline (PBS), pH 7.4. All the experiments were carried out with cell cultures of three days post-confluency between passages 102-108.

Animals—Inbred, male Wistar rats of 8-10 weeks age, weighing 230-270 g, were used for the study. They were maintained on a standard laboratory diet (Lipton's India, Calcutta, India) and water was given *ad libitum*.

In Vitro Study—Cisplatin-induced cytotoxicity in vero cells. Effect of IG, EG, or DZ: For the experimental study, vero cells from well established confluent monolayers were harvested using trypsin-EDTA. The isolated cells were suspended in modified Hank's balanced salt solution (HBSS), pH 7.4, centrifuged at 400 rpm for 5 min and then resuspended in HBSS to give 1×10^6 cells/ml. Then the cells were washed three times with HBSS to remove trypsin.

The time-course of the effect of cisplatin on the cytotoxicity in vero cells was investigated by incubating 1×10^6 cells with 50 μg/ml of cisplatin for 0, 30, 60, 90, and 120 min. Cell viability was determined by the tryphan blue exclusion (TBE) method (*23*) or lactate dehydrogenase (LDH) assay (*24*). Briefly, 10 μl of 4% tryphan blue was added to 90 μl of cell suspension, followed by examination with a hemocytometer under an inverted microscope. Cells which excluded the dye were considered viable and the data were expressed as percentage tryphan blue exclusion (% TBE). LDH release was assayed using LDH commercial kits "Monotest®" purchased from Boehringer Mannheim GmbH Diagnostics (Cat. no. 158186), Germany. Briefly, with 10 μl of medium, the oxidation of NADH (0.1 mM) was followed at 340 nm in the presence of pyruvate (1.0 mM) and 0.2 M Tris-hydrochloride buffer (pH 7.3), in a sample volume of 1.0 ml. Control cells not exposed to cisplatin were shaken gently for 10 min in 0.1% Triton X-100 and then triturated to lyse the cells. Percent cell death was calculated as the percentage of lactate dehydrogenase (% LDH) released by cisplatin compared to Triton-X 100 lysed control cells.

The concentration-dependent effect was studied by incubating vero cells (1×10^6) with 0, 10, 25, 50, 100, and 200 μg/ml of cisplatin for 1 h.

The effects of IG, EG, or DZ on the cisplatin-induced cytotoxicity and reduction of the GSH content in vero cells were studied by incubating 1×10^6 cells with various concentrations of IG, EG, or DZ in the presence or absence of cisplatin (50 μg/ml) for 1 h.

The GSH content was measured as non-protein sulfhydryl groups by Ellmann's method (*25*). Briefly, after cell lysis by sonication, the cell homogenate was treated with 0.9 ml distilled water, 1 ml phosphate buffer (pH 8) and 100 μl DTNB (10 mM). Absorbance was measured at 412 nm. GSH concentrations were expressed in terms of nmol/10^6 cells using a GSH molar extinction coefficient of 13,600 $M^{-1}\cdot cm^{-1}$.

Cisplatin-induced toxicity in rat renal cortical slices. Effect of IG, EG, or DZ: Renal cortical slices were prepared as described previously (*10, 11*). Briefly, after rats had been anesthetized with sodium pentobarbitone (60 mg/kg, i.p.), their kidneys were perfused transcardially with ice-cold normal saline to prevent contamination of the kidneys by blood. After removing the kidneys, renal cortical slices were prepared using a razor blade in a kidney holder device, the slice thickness being about 0.3-0.5 mm. Cortical slices of about 300 mg/sample were incubated for 120 min at 37°C, being shaken in an oxygen-saturated solution with the following composition (in mM): 0.074 PAH, 96.7 NaCl, 7.4 sodium phosphate buffer (pH 7.4), 40.0 KCl, 7.4 $CaCl_2$, and 10.0 lactic acid. Renal cortical slices were incubated in a sample volume of 4.0 ml with cisplatin in the presence or absence of a test compound for 120 min at 37°C. In control experiments, slices were incubated under identical conditions without cisplatin.

In renal cortical slices, lipid peroxidation was evaluated as malondialdehyde (MDA) production as described by Beuge and Aust (*26*). Briefly, at the end of the incubation, 100 mg of cortical slices was dried, weighed and homogenized using a teflon homogeniser (Remi Motors, Mumbai) at 3,000 rpm for 2 min in 5 ml of sodium phosphate buffer (pH 7.4) at 2°C; 1 ml homogenate was treated with 2 ml TBA reagent comprising 0.375% TBA, 15% TCA, and 0.25 N HCl. Samples were boiled for 15 min, cooled and then centrifuged. A blank was obtained by the addition of the reagent without TBA. The absorbance of the supernatant was spectrophotometrically measured at 535 nm. The MDA concentration was calculated using a molar extinction coefficient of MDA of 1.56×10^{-5} $M^{-1}\cdot cm^{-1}$ and expressed as nmol/g of tissue as reported earlier by us (*17*).

The GSH content in renal cortical slices was assayed as non-protein sulfhydryl groups by the method of Seldak and Lindsay (*27*). Briefly, a 3.75% w/v homogenate was prepared in a 20 mM EDTA solution. To 2.5 ml homogenate was added 2.5 ml of 10% TCA. After vigorous shaking samples were centrifuged. To 0.5 ml supernatant, 1 ml of 400 mM Tris buffer (pH 8.9) and 0.1 ml of 5 mM DTNB (in methanol) were added, and then the absorbance was spectrophotometrically measured at 412 nm after 20 min. The GSH concentration was calculated using a molar extinction coefficient of 13,600 $M^{-1}\cdot cm^{-1}$ and expressed as nmol/g of tissue.

The PAH content was measured according to the Bratton and Marshall method (*28*). At the end of the incubation, renal cortical slices were removed, blotted on filter paper, weighed, and then homogenized in 5 ml of 3% TCA per 100 mg of tissue at 2°C; 2.0 ml of the corresponding medium was treated similarly. After centrifugation for 10 min at 1,500 rpm, the PAH concentrations of the supernatant of the homogenate and the medium were measured. To 2.0 ml homogenate or incubation medium, 0.3 ml of 10 N H_2SO_4, 0.1 ml of 3 M NaCl, 0.1 ml of 0.4% $NaNO_2$, 0.1 ml of 2% ammonium sulphamate, and 0.1 ml of 0.2% naphthyl ethylene diamine hydrochloride reagent were added. The reagents were added at intervals of 5 min. After 60 min, the absorbance of the samples was measured at 556 nm. The concentrations in the tissue and medium were calculated using a PAH standard curve. The accumulation of PAH was expressed as the slice/medium (S/M) ratio (concentration of PAH per g of tissue/concentration of PAH per ml of medium).

In Vivo Study—The experiments were conducted according to our institutional regulations and national criteria for animal experiments. To examine the effect of IG, EG, or DZ on cisplatin nephrotoxicity, the rats were administered with various doses of IG, EG, or DZ i.p. 1 h before cisplatin 3 mg/kg. In separate experiments IG 10 mg/kg was administered i.p. 1 h after cisplatin. Blood was collected on day 5 to measure BUN and serum creatinine. BUN (diacetyl monoxime method) and serum creatinine (alkaline picrate method) kits were obtained from Ranbaxy Diagnostics, New Delhi, India. Body weight was determined once a day.

Statistical Analysis—The results are expressed as means ± SD for *in vitro* experiments or means ± SE for *in vivo* experiments. For comparison, one-way ANOVA followed by Student's Newman-Keuls test was used. Statistical significance was set at $p<0.05$. All the statistical analysis was performed using an SPSS-PC version 3.1 computer package.

RESULTS

Effect of IG, EG, or DZ on Cisplatin-Induced Cytotoxicity in Vero Cells—In vero cells, the cytotoxicity of cisplatin can be measured as tryphan blue exclusion (% TBE) and the release of lactate dehydrogenase (% LDH) from the cells. The tryphan blue excluded by the cells which are viable and a decrease in % TBE are indicators of cytotoxicity. An increase in LDH released from cells into the medium also indicates cytotoxicity.

Figure 1, A and B, shows the time-dependent cytotoxicity induced by cisplatin. Incubation of vero cells (1×10^6) with 50 μg/ml cisplatin resulted in a time-dependent decrease in % TBE, and at 120 min the viability was only about 50%. The release of LDH also increased significantly after 60 min. Figure 1, C and D, shows the concentration-dependent cytotoxicity of cisplatin. Incubation of vero cells (1×10^6) with various concentrations of cisplatin resulted in concentration-dependent decreases in % TBE and % LDH release. Incubation of cisplatin with vero cells (1×10^6) for 1 h resulted in a reduction of the GSH content in a concen-

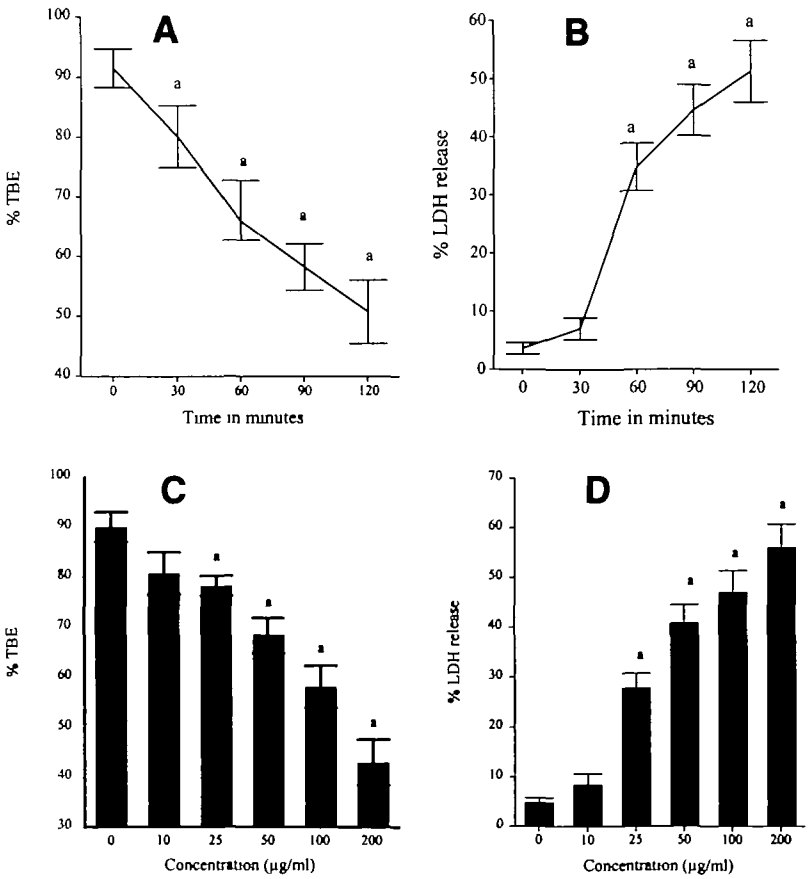

Fig. 1. **A: Time-course of the effect of cisplatin on cytotoxicity in vero cells.** Vero cells (1×10^6) were incubated with 50 μg/ml of cisplatin for 0, 30, 60, 90, and 120 min, and then the cytotoxicity was measured as percentage lactate dehydrogenase release (% LDH). The results are expressed as means ± SD ($n=5$). $^a p<0.05$ compared to control. **B: Time-course of the effect of cisplatin on cytotoxicity in vero cells.** Vero cells (1×10^6) were incubated with 50 μg/ml of cisplatin for 0, 30, 60, 90, and 120 min, and then the cytotoxicity was measured as percentage tryphan blue exclusion (% TBE). The results are expressed as means ± SD ($n=5$). $^a p<0.05$ compared to control. **C: Concentration-dependent effect of cisplatin on cytotoxicity in vero cells.** Vero cells (1×10^6) were incubated with 0, 10, 25, 50, 100, and 200 μg/ml of cisplatin for 1 h, and then the cytotoxicity was measured as percentage tryphan blue exclusion (% TBE). The results are expressed as means ± SD ($n=5$). $^a p<0.05$ compared to control. **D: Concentration-dependent effect of cisplatin on cytotoxicity in vero cells.** Vero cells (1×10^6) were incubated with 0, 10, 25, 50, 100, and 200 μg/ml of cisplatin for 1 h, and then the cytotoxicity was measured as percentage lactate dehydrogenase (% LDH) release. The results are expressed as means ± SD ($n=5$). $^a p<0.05$ compared to control.

tration-dependent manner (Fig. 2).

The effect of IG, EG, or DZ on the cytotoxicity induced by cisplatin (50 μg/ml) is shown in Table I. The viability, as measured as % TBE, of cells treated with IG, EG, and DZ at 1.0 mM was about 83, 80, and 81%, respectively, compared to 68% for cisplatin-treated cells. IG-treated cells showed only 17% LDH release, EG- and DZ-ones showing 28 and 26% release, respectively, at 1.0 mM (cisplatin treated = 41% release). Partial protection was also observed at 0.5 mM. All were inactive at 0.1 mM.

Cisplatin (50 μg/ml) reduced the GSH level to 5.9 ± 0.54 nmol/10^6 cells. In the control, the GSH level was 9.0 ± 0.51 nmol/10^6 cells. None of the test compounds showed any protection against GSH depletion (Table I).

Effect of IG, EG, or DZ on Cisplatin-Induced Toxicity in Renal Cortical Slices—Incubation of renal cortical slices with various concentrations of cisplatin resulted in a concentration-dependent increase of MDA, and decreases in GSH and PAH uptake (Figs. 3, 4, and 5).

Table II shows the effects of IG, EG, and DZ on the cisplatin-induced increase in the MDA level and the

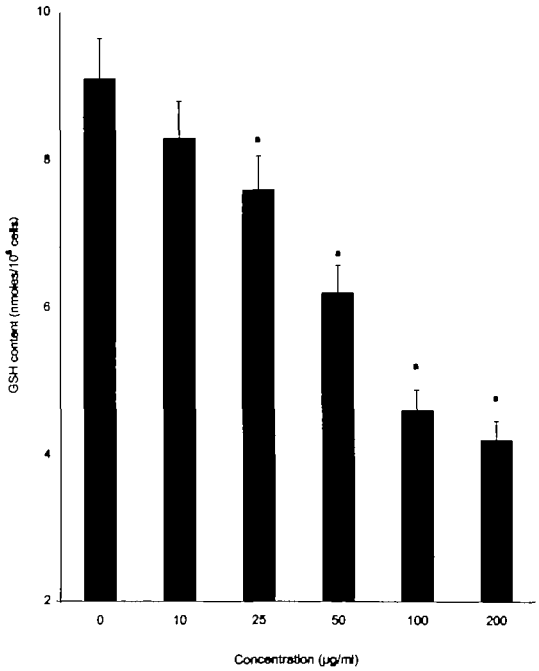

Fig. 2. **Concentration-dependent effect of cisplatin on the glutathione (GSH) content in vero cells.** Vero cells (1×10^6) were incubated with 0, 10, 25, 50, 100, and 200 μg/ml of cisplatin for 1 h, and then the cytotoxicity was measured as non-protein sulfhydryl groups. The results are expressed as means ± SD ($n = 5$). $^a p < 0.05$ compared to control.

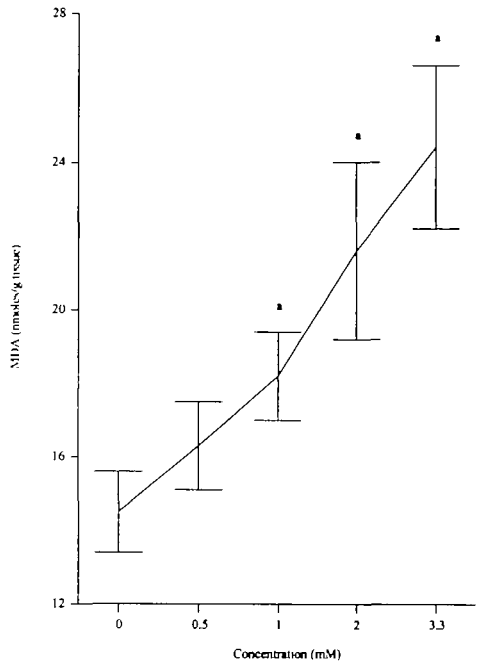

Fig. 3. **Concentration-dependent effect of cisplatin on the malondialdehyde (MDA) content in renal cortical slices.** Renal cortical slices were incubated with 2.0 mM cisplatin for 120 min. The slices were dried, weighed, and then homogenised. Then 1.0 ml homogenate was boiled with 2.0 ml TBA reagent, cooled, and centrifuged, and then the absorbance at 535 nm was measured. The MDA concentration was calculated using a molar extinction coefficient of MDA of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ and expressed as nmol/g of tissue. The results are expressed as means ± SD ($n = 4$). $^a p < 0.05$ compared to control.

TABLE I. **Effects of isoeugenol (IG), eugenol (EG), and dehydrozingerone (DZ) against cisplatin-induced cytotoxicity in vero cells.** Vero cells (1×10^6) were incubated with IG, EG, or DZ in the presence or absence of cisplatin (50 μg/ml) for 1 h. The percentage tryphan blue exclusion (% TBE), percentage lactate dehydrogenase (% LDH) release, and glutathione (GSH) were measured as described under "MATERIALS AND METHODS." ND, not determined. The results are expressed as means ± SD ($n = 5$).

Drug	Concentration (mM)	% TBE		% LDH release		GSH (nmol/10^6 cells)	
		Cisplatin-treated cells	Control cells	Cisplatin-treated cells	Control cells	Cisplatin-treated cells	Control cells
Cisplatin	–	68 ± 4[a]	90 ± 3	41 ± 4[a]	4.9 ± 0.9	5.9 ± 0.5[a]	9.0 ± 0.5
Cisplatin + IG	0.1	65 ± 5[a]	92 ± 5	39 ± 4[a]	5.2 ± 1.0	ND	ND
	0.5	80 ± 4[b]	87 ± 6	26 ± 4[a,b]	4.6 ± 1.1	5.1 ± 0.7[a]	8.7 ± 0.6
	1.0	83 ± 5[b]	87 ± 7	17 ± 3[a,b]	6.2 ± 1.5	6.2 ± 0.6[a]	9.1 ± 0.7
Cisplatin + EG	0.1	69 ± 5[a]	86 ± 7	42 ± 6[a]	6.1 ± 0.8	ND	ND
	0.5	76 ± 5[a]	90 ± 8	30 ± 5[a,b]	6.5 ± 1.2	5.6 ± 0.6[a]	9.5 ± 1.0
	1.0	80 ± 5[b]	89 ± 6	27 ± 4[a,b]	5.9 ± 0.9	5.8 ± 0.6[a]	9.4 ± 0.8
Cisplatin + DZ	0.1	66 ± 4[a]	89 ± 6	42 ± 4[a]	6.1 ± 0.9	ND	ND
	0.5	79 ± 5[a,b]	92 ± 5	29 ± 4[a,b]	5.7 ± 0.8	6.0 ± 0.8[a]	8.9 ± 0.8
	1.0	81 ± 6[a,b]	96 ± 7	27 ± 4[a,b]	4.6 ± 1.1	6.3 ± 0.8[a]	9.2 ± 0.9

$^a p < 0.05$ compared to control. $^b p < 0.05$ compared to cisplatin-treated.

decrease in the GSH content. Cisplatin (2.0 mM) increased the MDA level to 21.6±2.3 nmol/g tissue compared to the control (14.5±1.1 nmol/g tissue). IG, EG, and DZ each inhibited the cisplatin-induced increased MDA level at 0.5 and 1.0 mM. At 0.5 and 1.0 mM, IG was slightly more active than EG and DZ. All the compounds were found to be inactive at 0.1 mM. The rank order of activity was: IG> EG>DZ. Cisplatin (2.0 mM) reduced the GSH level to 99±11 nmol/g tissue (control=138±19). None of the test compounds showed any protection against GSH depletion (Table II).

Table II also shows the effects of IG, EG, and DZ on cisplatin-induced inhibition of PAH uptake in renal cortical slices. Cisplatin (2.0 mM) decreased the PAH level to 2.00±0.35 (slice/medium ratio) compared to the control (3.23±0.27, slice/medium ratio). IG showed significant protection against decreased PAH uptake at 0.5 and 1.0

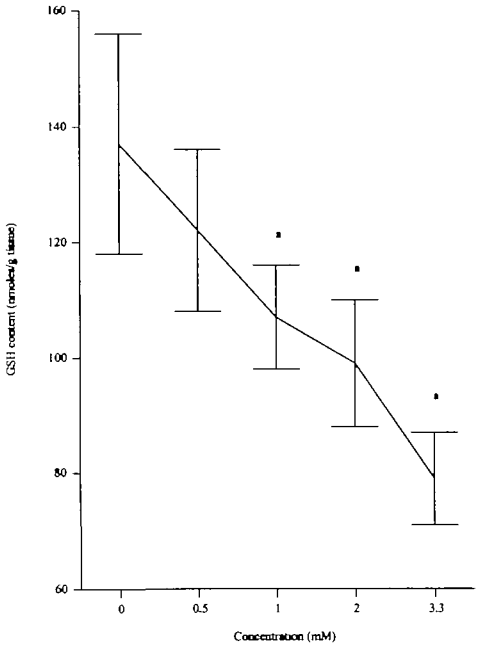

Fig. 4. **Concentration-dependent effect of cisplatin on the glutathione (GSH) content in renal cortical slices.** Renal cortical slices were incubated with 2.0 mM cisplatin for 120 min. The slices were dried, weighed and then homogenised. Then 2.5 ml homogenate was treated with 2.5 ml 10% TCA, and centrifuged after vigorous shaking. To 0.5 ml supernatant, 1 ml of 400 mM Tris buffer (pH 8.9) and 0.1 ml of 5 mM DTNB (in methanol) were added, and then the absorbance was spectrophotometrically measured at 412 nm after 20 min. The GSH concentration was calculated using a molar extinction coefficient of 13,600 $M^{-1} \cdot cm^{-1}$ and expressed as nmol/g of tissue. The results are expressed as means±SD ($n=4$). $^{a}p<0.05$ compared to control.

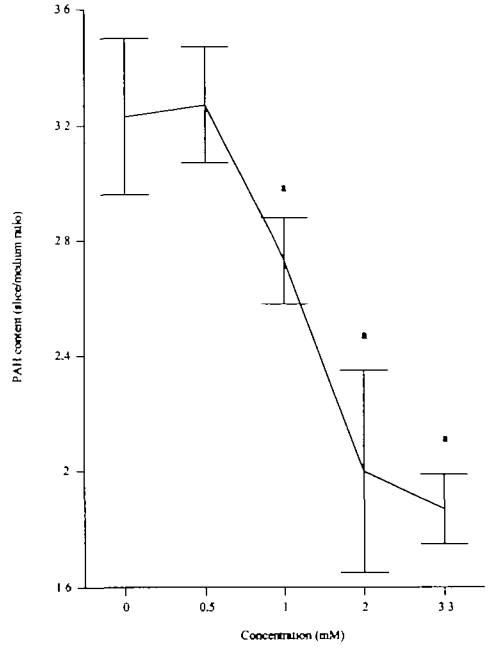

Fig. 5. **Concentration-dependent effect of cisplatin on *p*-aminohippurate (PAH) uptake in renal cortical slices.** Renal cortical slices were incubated with 2.0 mM cisplatin for 120 min. The slices were dried, weighed, and then homogenised. Then the slices were treated with Bratton and Marshall reagent. After 60 min, the absorbance of the samples was measured at 556 nm. The concentrations in the tissue and medium were calculated using a PAH standard curve. The accumulation of PAH was expressed as the slice/medium ratio (concentration of PAH per g of tissue/concentration of PAH per ml of medium). The results are expressed as means±SD ($n=4$). $^{a}p<0.05$ compared to control.

TABLE II. **Effects of isoeugenol (IG), eugenol (EG), and dehydrozingerone (DZ) against cisplatin-induced cytotoxicity in renal cortical slices.** Renal cortical slices were incubated with IG, EG, or DZ in the presence or absence of cisplatin (2 mM) for 120 min. The malondialdehyde (MDA), glutathione (GSH), and *p*-aminohippurate (PAH) were measured as described under "MATERIALS AND METHODS." The results are expressed as means±SD ($n=4$).

Drug	Concentration (mM)	MDA (nmol/g tissue)		GSH (nmol/g tissue)		PAH (slice/medium ratio)	
		Cisplatin-treated slices	Control slices	Cisplatin-treated slices	Control slices	Cisplatin-treated slices	Control slices
Cisplatin	–	21.6±2.3[a]	14.5±1.1	99±11[a]	137±19	2.0±0.4[a]	3.2±0.3
Cisplatin+IG	0.1	20.2±1.9[a]	14.9±1.2	106±12[a]	141±15	2.2±0.3[a]	3.2±0.2
	0.5	16.8±1.4[a,b]	13.8±1.4	103±10[a]	138±17	2.6±0.2[a,b]	3.1±0.2
	1.0	14.0±1.5[b]	13.6±1.3	116±11[a]	141±16	3.0±0.2[b]	3.2±0.3
Cisplatin+EG	0.1	19.8±2.0[a]	14.2±1.4	96±10[a]	141±15	2.2±0.3[a]	3.4±0.4
	0.5	17.6±1.3[a,b]	12.9±1.3	102± 9[a]	130±12	2.5±0.4[a,b]	3.2±0.3
	1.0	15.2±2.1[b]	12.8±1.3	100± 9[a]	136±15	2.7±0.2[a,b]	3.3±0.3
Cisplatin+DZ	0.1	20.2±1.8[a]	14.6±1.2	101±10[a]	143±11	1.9±0.2[a]	3.1±0.3
	0.5	18.1±1.3[a]	13.8±1.4	99±11[a]	133±18	2.3±0.3[a]	3.3±0.3
	1.0	16.9±1.3[b]	14.1±1.6	109± 9[a]	150±15	2.8±0.3[a,b]	3.4±0.3

$^{a}p<0.05$ compared to control. $^{b}p<0.05$ compared to cisplatin-treated.

TABLE III. **Effects of isoeugenol (IG), eugenol (EG), and dehydrozingerone (DZ) against cisplatin-induced nephrotoxicity in rats.** IG, EG, or DZ was administered i.p. 1 h prior to cisplatin 3 mg/kg i.p. On day 5, blood was collected to measure blood urea nitrogen (BUN) and serum creatinine. Body weight was recorded daily. The results are expressed as means ± SE ($n=10$).

Drug	Dose (mg/kg)	Change in body weight (g)		BUN (mg/100 ml)		Serum creatinine (mg/100 ml)	
		Cisplatin-treated rats	Control rats	Cisplatin-treated rats	Control rats	Cisplatin-treated rats	Control rats
Cisplatin	–	−10.8±1.9[a]	11.9±1.2	48.1±1.7[a]	19.2±1.1	1.29±0.07[a]	0.60±0.03
Cisplatin+IG	1.0	−9.6±2.2[a]	10.8±1.9	45.2±2.8[a]	17.3±1.6	1.19±0.07[a]	0.62±0.04
	5.0	−10.6±1.8[a]	9.8±1.6	44.6±4.0[a]	18.2±2.1	1.13±0.09[a]	0.66±0.05
	10.0	−3.4±1.7[a,b]	12.0±2.5	34.7±3.0[a,b]	19.1±1.8	0.86±0.05[a,b]	0.61±0.06
Cisplatin+EG	1.0	−10.5±3.5[a]	11.5±3.1	48.8±3.3[a]	17.9±1.6	1.26±0.16[a]	0.68±0.09
	5.0	−8.6±3.0[a]	12.6±4.9	46.1±3.9[a]	18.1±1.9	1.26±0.11[a]	0.61±0.05
	10.0	−7.8±4.0[a]	11.5±3.1	43.4±4.1[a]	18.9±1.6	1.19±0.09[a]	0.66±0.09
Cisplatin+DZ	1.0	−9.9±3.6[a]	12.3±4.6	47.3±4.0[a]	18.0±2.1	1.30±0.14[a]	0.71±0.09
	5.0	−9.5±2.0[a]	11.8±2.8	45.8±4.1[a]	16.8±2.8	1.31±0.12[a]	0.59±0.06
	10.0	−8.5±2.5[a]	10.6±2.6	43.6±3.9[a]	18.4±1.2	1.18±0.08[a]	0.63±0.05

[a] $p<0.05$ compared to control. [b] $p<0.05$ compared to cisplatin-treated.

mM. EG and DZ were active only at 1.0 mM. None of the compounds were active at 0.1 mM. The rank order of activity was: IG > DZ > EG.

Effect of IG, EG, or DZ against Cisplatin-Induced Nephrotoxicity in Rats—Effect on body weight: When animals were given cisplatin 3 mg/kg i.p. the weight of the animals decreased significantly (Table III). There was an average decrease of 10.8±1.9 g on day five compared to control animals that gained 11.9±1.2 g during the same period. When the animals were given IG, EG, or DZ the body weight was the same as the control level. IG (10 mg/kg, i.p.) showed significant protection against the cisplatin-induced reduction in the body weight (−3.4±1.7 g), *i.e.* 34% protection. However, EG and DZ (1-10 mg/kg, i.p.) did not show any significant protection.

Effects on BUN and Serum Creatinine—The administration of cisplatin 3 mg/kg i.p. resulted in a significant increase in the BUN level (Table III). The BUN level increased to 48.1±1.7 mg/100 ml compared to 19.2±1.1 mg/100 ml in control animals. When IG, EG, or DZ was given 1 h before cisplatin 3 mg/kg i.p. only IG 10 mg/kg i.p. protected the BUN level (34.7±3.0 mg/100 ml), the protection being 46%. Similar results were obtained for serum creatinine (Table III). When cisplatin (3.0 mg/kg, i.p.) was given the serum creatinine level increased to 1.29±0.07 mg/100 ml compared to the control value of 0.60±0.03 mg/100 ml. IG (10 mg/kg, i.p.) 1 h before cisplatin protected against the elevation of serum creatinine (0.86± 0.05 mg/100 ml), the protection being 62%.

Multiple doses of IG (10 mg/kg i.p. ×3 days or 5 days, or an alternate days) did not improve the cisplatin-induced renal damage (data not shown). None of the compounds were active orally (data not shown). IG 10 mg/kg i.p. 1 h after cisplatin did not show any protection (data not shown).

DISCUSSION

The present study showed that cisplatin causes cytotoxicity in vero cells, as measured as % TBE, % LDH release, and GSH content. The protective properties of three structurally related compounds: IG, EG, and DZ, which are potent antioxidants, against cisplatin-induced toxicity were studied. IG was the most active compound, followed by EG and then DZ, in preventing cell death induced by cisplatin. Several investigators have used an *in vitro* system to study cisplatin-induced cytotoxicity (*29-32*). Cisplatin is known to cause cytotoxicity in kidney cells due to oxidative injury (*32, 33*). Recently, Tsutsumishita and coworkers reported the involvement of hydrogen peroxide in cisplatin-induced nephrotoxicity in outer medullary cortical tubule cells (*34*). It has been suggested that cisplatin-induced cytotoxicity may be due to the peroxidation of cell membranes (*32*). Moreover, many antioxidants prevented cisplatin-induced cytotoxicity (*32, 33*). In the present study, none of the compounds were able to prevent the cisplatin-induced reduction of the GSH content.

Several investigators have shown that cisplatin nephrotoxicity is associated with lipid peroxidation in renal cortical slices. Recently, Inselmann and coworkers suggested that cisplatin-induced lipid peroxidation inhibits the uptake of PAH by rat renal cortical slices (*11*). Antioxidants such as *N,N′*-diphenyl-*p*-phenylenediamine (*35*), and a *Gingko biloba* extract (*11*) partially reversed cisplatin-induced lipid peroxidation and inhibition of PAH uptake. Cystone extract, a polyayurvedic herbal preparation, also inhibited cisplatin-induced lipid peroxidation in rat renal cortical slices and nephrotoxicity in rats (*10*). The present study also showed that cisplatin causes lipid peroxidation, decreases the GSH content and inhibits the uptake of PAH by renal cortical slices. IG was most active in inhibiting lipid peroxidation and it also reversed the inhibition of PAH uptake. DZ was active, but lesser than EG. GSH depletion was shown to enhance cisplatin-induced lipid peroxidation (*36*). None of the compounds were able to prevent the reduction of the GSH content.

Many antioxidants show protection against cisplatin-induced renal toxicity *in vivo* (*4, 6, 12, 14*). In the present study, cisplatin significantly decreased the body weight, and elevated BUN and serum creatinine in rats. Among the antioxidants studied, only IG was found to be active. IG 10 mg/kg i.p. 1 h before cisplatin reduced cisplatin-induced renal toxicity in rats. No protection was observed when IG was given 1 h after cisplatin (data not shown). Multiple doses (2 mg/kg i.p. ×5 days) and oral treatment did not show any protection (data not shown). Lower doses than 10 mg/kg i.p. did not show any protection. The induction of nephrotoxicity by cisplatin is assumed to be a fast process involving reaction with proteins in renal tubules (*37, 38*). This renal damage is caused in the first hour after adminis-

tration (*39*). Hence, it is important that protective agents must be present at sufficient concentrations in the renal tissue before the damage occurs. This further explains why for IG to be effective, it must be given in advance of cisplatin. This may also explain why IG did not show improved protection when given in multiple doses or 1 h after cisplatin administration.

In conclusion, the present study showed that IG, a naturally occurring phenolic antioxidant, is significantly active in protecting against cisplatin-induced toxicity both *in vitro* and *in vivo*. Protection was observed only when IG was given before cisplatin. Other antioxidants studied, like EG and DZ, were active *in vitro* but inactive *in vivo*. The facts that IG is an antioxidant, and that it prevents cisplatin-induced toxicity *in vitro* and *in vivo* make the present conclusions interesting and provide impetus for further studies.

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