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Protective agent, erdosteine, against cisplatin-induced hepatic oxidant injury in rats

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Abstract

Cisplatin, one of the most active cytotoxic agents against cancer, has several toxicities. Hepatotoxicity is one of them occurred during high doses treatment. The aim of this study was to determine the effects of erdosteine against cisplatin-induced liver injury through tissue oxidant/antioxidant parameters and light microscopic evaluation. The rats were randomly divided into three groups: control ($n = 5$), cisplatin (10 mg/kg, $n = 6$) and cisplatin+erdosteine (50 mg/kg/day oral erdosteine, $n = 8$) groups. The rats were sacrificed at the 5th day of cisplatin treatment. The liver tissues were examined with light microscopy and oxidant/antioxidant biochemical parameters. The malondialdehyde (MDA) and nitric oxide (NO) levels were increased in the cisplatin group in comparison with the control and cisplatin+erdosteine groups ($p < 0.05$). There was no significant difference in MDA and NO levels between control and cisplatin+erdosteine groups. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were higher in cisplatin+erdosteine group than cisplatin group ($p < 0.05$). However, the CAT and GSH-Px activities were significantly lower in cisplatin group than in control group ($p < 0.05$). The light microscopic examination revealed that cytoplasmic changes especially around cells of central vein were observed in cisplatin group. Hepatocellular vacuolization was seen in these cells. In the cisplatin plus erdosteine group, a decrease in cytoplasmic changes with the hepatocytes and sinusoidal dilatations around cells of central vein were noticed in as compared to cisplatin group. In the light of microscopic and biochemical results, it was concluded that cisplatin-induced liver damage in high dose and erdosteine prevented this toxic side effect by the way of its antioxidant and radical scavenging effects. (Mol Cell Biochem **278:** 79–84, 2005)

Key words: cisplatin, light microscopy, erdosteine, liver, oxidant/antioxidant

Introduction

Cisplatin, one of the most active antineoplastic drugs against ovarian, testicular and head and neck cancers [1, 2] has limited usage due to its undesirable side effects, nephrotoxicity and neurotoxicity being the most severe and dose-limiting ones [3, 4]. However, dose dependent cisplatin-induced hepatotoxicity can alter the clinical situation of patients. The alterations in the kidney and liver functions induced by cisplatin are closely associated with an increase in lipid peroxidation in the tissues [5, 6]. Oxidative damage to membrane lipids and other cellular components is believed to be

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a major factor in the cisplatin toxicity [7]. Cisplatin causes excessive reactive oxygen species (ROS) generation which exist normally in the cellular environment. However, endogenous antioxidant systems prevent their toxic effects to living organism in normal healthy conditions. Hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\bullet}OH$) are thought to be produced during oxidative stress [8, 9]. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) are endogenous antioxidants which play role in prevention of oxidative injury [10]. The other radical producing mechanism is nitric oxide (NO) which reacts with $O_2^{\cdot -}$ to form peroxinitrite, a toxic agent to the cellular components [11]. Thus many antioxidant agents have been studied in experimental and clinical studies to reduce or prevent cisplatin-induced nephrotoxicity [12, 13]. Erdosteine, widely orally used mucolytic and expectorant in clinics, has two blocked thiol groups. The active metabolites of erdosteine exhibit free radical scavenging activity via its sulphydryl groups released following erdosteine catabolism in the liver [14–16]. It was demonstrated that oral erdosteine treatment prevents lipid peroxidation in various tissues of rats which were treated with doxorubicin [16], bleomisin [17] and rotenone [18] Therefore, the aim of the present study was to investigate the effects of erdosteine treatment against cisplatin-induced liver damage through oxidant/antioxidant parameters and light microscopic evaluation.

Materials and methods

Animals and experimental procedure

Adult male Wistar albino rats were used in this study. The animals were fed with commercial standard diet and water *ad libitum* during the experimental period, besides keeping them in the room ambient temperature, and 12:12 h light and dark cycles were maintained. All animals were housed for acclimatization for 1 week before the experiments. This research was performed in accordance with "Guide for the care and Use of Laboratory Animals, DHEW Publication No. (NIH) 85–23, 1985".

The rats were randomly divided into three groups: untreated control ($n = 5$), i.p dose of 10 mg/kg cisplatin (Cisplatinum Ebewe, 0.5 mg ml^{-1}) injected (6 rats were alive after 5th day of the treatment and one rat was dead before 5th day, $n = 6$) and starting 2 days before cisplatin injection 50 mg/kg/day [13] oral erdosteine (Ilsan-Iltas, Turkey) [7] treated cisplatin+erdosteine ($n = 8$) groups. The rats were sacrificed at the 5th day of cisplatin treatment. The rats were anesthetized with a cocktail of i.p. ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) before sacrifice processing. Then, the liver samples were removed from the animals and pieced into two for biochemical and light microscopic examinations. The tissues were stored at $-30\,^{\circ}C$ until biochemical analysis.

Homogenization and determination of protein

After weighing the liver tissue, homogenate, supernatant and extracted samples were prepared [19] and the following determinations were made on the samples using commercial chemicals supplied by Sigma (St. Louis, USA). Tissues were homogenized in four volumes of ice-cold Tris-HCl buffer (50 milimolar, pH 7.4) using a glass Teflon homogenizer (Ultra Turrax IKA T25 Basic, Germany) after cutting of the livers into small pieces with a scissors (for 2 min at 5000 rpm). Analysis of malondialdehyde (MDA), nitric oxide (NO) and protein levels were carried out at this stage. The homogenate was then centrifuged at $5000 \times g$ for 60 min to remove debris. Clear supernatant fluid was taken, analysis of CAT and GSH-Px activities as well as protein concentration was carried out in this stage. The supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (5/3, volume per volume [v/v]). After centrifugation at $5000 \times g$ for 30 min, the clear upper layer (the ethanol phase) was taken and used in the analysis of SOD activity and protein assays. Protein measurements were analyzed in homogenate, supernatant and extracted samples according to the method of Lowry *et al.* [20].

Determination of catalase activity

Catalase (EC 1.11.1.6) activity was determined according to Aebi's method [21]. The principle of the assay is based on the determination of the rate constant (s^{-1}, k) or the H_2O_2 decomposition rate at 240 nm. Results were expressed as k/g tissue protein.

Determination of glutathione peroxidase activity

Glutathione peroxidase (EC 1.6.4.2) activity was measured by the method of Paglia and Valentine [22]. The enzymatic reaction in the tube, which is containing following items: NADPH, reduced glutathione, sodium azide, and glutathione reductase, was initiated by addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer. Results were expressed as U/g tissue protein.

Determination of tissue malondialdehyde level

The tissue MDA level was determined by a method [23] based on the reaction with thiobarbituric acid (TBA) at 90°–$100\,^{\circ}C$.

In the TBA test reaction, MDA or MDA-like substances and TBA react with the production of a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90 °C for 15 min. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed according to the standard graphic, which was prepared by the measurements done with a standard solution (1,1,3,3-tetramethoxypropane). Results were expressed as nanomol/g wet tissue.

Determination of superoxide dismutase activity

Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun *et al.* [24]. The principle of the method is based on the inhibition of nitroblue-tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate and the results were expressed as U/mg protein.

Nitric oxide determination

As NO measurement is very difficult in biological specimens, tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. The method for liver nitrite and nitrate levels was based on the Griess reaction [25]. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite+nitrate) was measured spectrophotometrically at 545 nm after conversion of nitrate to nitrite by copporized cadmium granules. A standard curve was established with a set of serial dilutions (10^{-8}–10^{-3} mol/l) of sodium nitrite. Linear regression was done by using the peak area from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. NO results were expressed as micromol/g wet tissue.

Histological evaluation

For light microscopy, liver tissue were removed and fixed in %10 neutral buffered formalin. The tissues were embedded in paraffin. The paraffin blocks were cut in 5 μm thick. The sections were stained with hematoxylin-eosin (H&E) and all sections were examined under light microscope.

Statistical analysis

Data were analyzed by using a commercially available statistics software package (SPSS® for Windows v. 9.0, Chicago, USA). Distribution of the groups was analyzed with one sample Kolmogrov-Smirnov test. All groups showed normal distribution, so that parametric statistical methods were used to analyse the data. One-way ANOVA test was performed and post hoc multiple comparisons were done with LSD. Results were presented as means ± S.E.M. p-values <0.05 were regarded as statistically significant.

Results

Biochemical results

The biochemical results are expressed in Table 1. The MDA level in the cisplatin group was significantly higher than in other groups ($p < 0.05$). There was no significant difference in MDA levels between control and cisplatin+erdosteine groups. The NO level of cisplatin group was increased in comparison with control and cisplatin+erdosteine groups ($p < 0.05$). The activities of SOD, CAT and GSH-Px were increased in cisplatin+erdosteine group in comparison with cisplatin group ($p < 0.05$). Also, the CAT and GSH-Px activities were significantly lower in cisplatin group than control group ($p < 0.05$).

Table 1. The levels of malondialdehyde (MDA) and nitric oxide (NO) with acitivities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in liver tissue of all groups (means ± S.E.M.)

	CAT (k/g prot.)	SOD (U/mg prot.)	GSH-Px (U/g prot.)	MDA (nmol/g wet tissue)	NO (μmol/g wet tissue)
Control ($n = 5$)	0.753 ± 0.100^b	0.192 ± 0.009	0.780 ± 0.034^b	14.76 ± 1.32^b	0.343 ± 0.056^b
Cis ($n = 6$)	0.450 ± 0.027^a	0.160 ± 0.011	0.406 ± 0.061^a	21.34 ± 1.27^a	0.633 ± 0.058^a
Cis+Erdosteine ($n = 8$)	0.672 ± 0.034^b	0.212 ± 0.012^b	0.627 ± 0.066^b	15.43 ± 0.48^b	0.468 ± 0.047^b

Cis: Cisplatin.
$^a p < 0.05$ in comparison with control group.
$^b p < 0.05$ in comparison with cisplatin group.

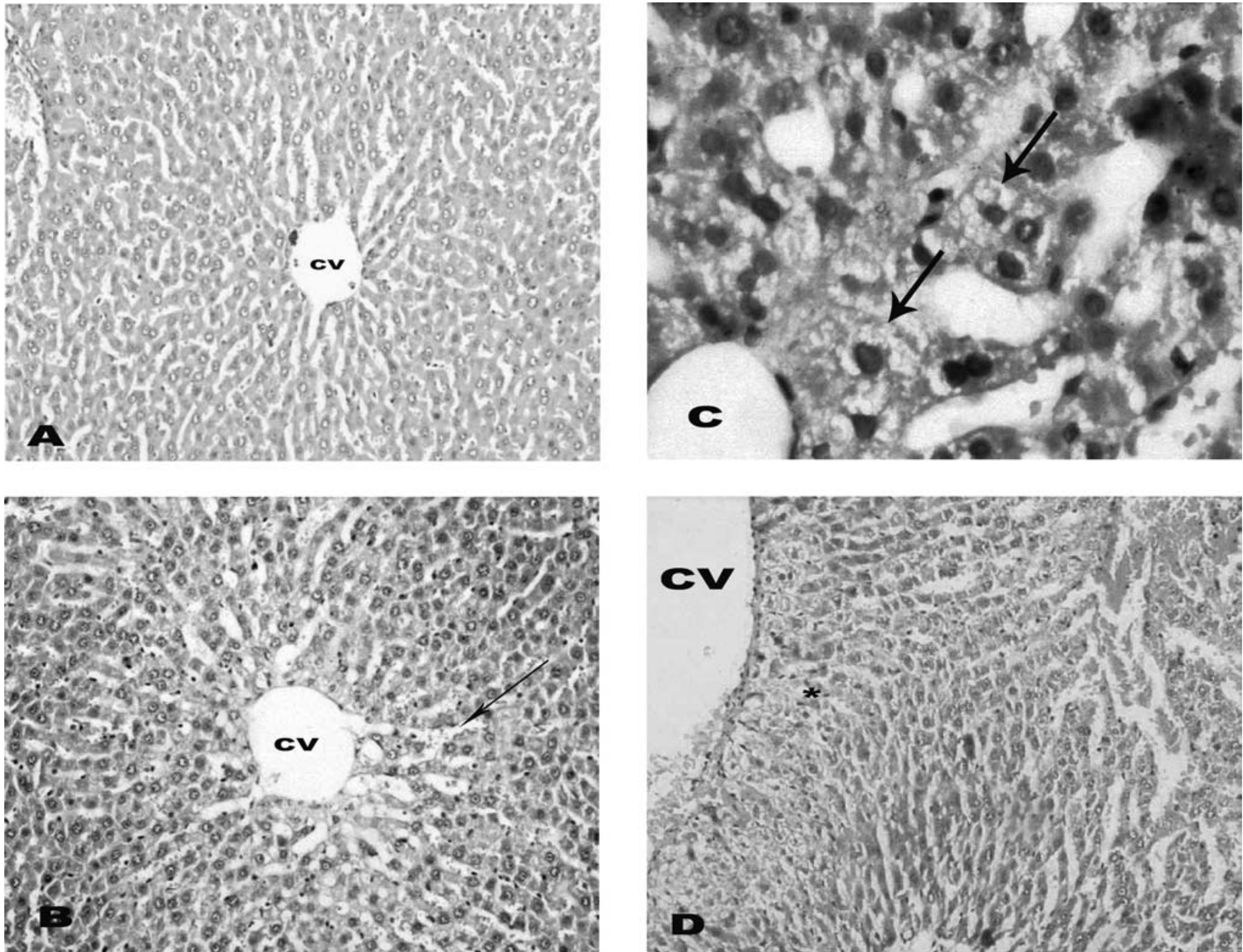

Fig. 1. (A) Light micrograph of rat liver section from control group. HE (original magnification, X100). (B) Liver of the cisplatin group. Rats were treated with 10 mg/kg cisplatin. Note to sinusoidal dilatation and congestion (arrow). HE (original magnification, X100). (C) Liver of the cisplatin group. Arrows indicates heptocellular vacuolization. HE (original magnification, X400). (D) Liver histology after cisplatin + erdosteine treatments of rats. Note, decreased hapatocellular vacuolization and congestion (asterisk). HE (original magnification, X100). Central vein (CV).

Light microscopic examination results

In control group, hepatocyte plates were normal and fibrosis, ductal dilatation and proliferation or inflammatory infiltrations were not observed. In addition, in this group, hepatocellular vacuolization was not seen. The lobuli were regular in shape. (Fig. 1A).

In cisplatin group, parenchyma around the central vein shows structural modifications. Cytoplasmic changes were observed especially in the cells around the central vein. Hepatocellular vacuolizations were also seen in these cells. Focal sinusoidal dilatation and congestion around central vein were slightly more visible in cisplatin group than in control group. In this group, clusters of inflammatory cells surrounding portal area (mostly plasma cells and lymphocyte) were seen (Figs. 1B and C).

In cisplatin plus erdosteine group, marked decreases in cytoplasmic changes of the hepatocytes and sinusoidal dilatations around central vein were noticed when compared to cisplatin group. The inflammatory cell infiltration in the portal area was less in cisplatin plus erdosteine group than that in cisplatin group. It was striking that the histological appearance of parenchyma in cisplatin plus erdosteine group was quite comparable with that of control group (Fig. 1D).

In all three groups, the hepatocytes with necrotic morphology were rare and foci of inflammatory cells in parenchyma were absent under light microscopic examinations.

Discussion

Hepatotoxicty is a rare side effect of cisplatin. However, it is known that cisplatin is significantly taken up in human

liver [26]. Some reports suggest that cisplatin-induced hepatotoxicity may be dose-related [26, 27]. The nephrotoxicity of cisplatin is well documented as the most important dose-limiting factor in cancer chemotherapy, while, at standard doses, hepatotoxicity rarely occurs and does not receive much attention. However, at high doses of cisplatin, hepatotoxicity does occur [28]. Hepatotoxicity was reported in high dose of cisplatin administration in rats [6]. A previous study indicates that cisplatin-induced hapatotoxicity occurs when high doses (>100 μmol/kg) are given in mice [28]. Zicca *et al.* showed that the high dose of cisplatin (7.5 mg/kg) administered to rats caused an evident liver damage characterized by significant increase of glutamic oxalacetic transaminase and γ-glutamyl transpeptidase plasma activities [26]. In our experiments, rats were treated with 10 mg/kg high dose of cisplatin and hepatotoxicity was apparent at this dose of cisplatin-treated rats.

Jie *et al.* have shown that necrotic cells were rarely found in cisplatin-induced liver injury. In the present study, we did not observe any necrotic cells in liver either. The same authors demonstrated that cisplatin produce more apoptotic cells, rather than necrotic cell, in liver [28]. In contrary, in our study, we did not see apoptotic lesions. Apoptosis was scarcely observed in the normal quiescent hepatocytes after cisplatin injection. However, in another study apoptosis has been observed at an early phase in regenerating liver following partial hepatectomy, implying that cisplatin-induced apoptosis only occurs in the primed hepatocytes [29], which is consistent with our findings.

Zicca *et al.* [26] have shown that when rats were treated with 7.5 mg/kg cisplatin, parenchyma shows structural modifications in hepatic lobules; the lobules show an irregular shape and central veins show normal structure. Consistently, our findings were similar. In addition, observed hepatocellular vacuolization, sinusoidal dilatation and congestion were observed in our study. The treatment with erdosteine (50 mg/kg/day oral erdosteine) considerably reversed the histological damage induced by cisplatin injection.

The reactive oxygen species (ROS) are thought to be the mechanism of cisplatin-induced cellular injury. The balance between oxidant and antioxidant system seemed to be disturbed in our study due to cisplatin injection. The high dose of cisplatin administration caused a hepatic lipid peroxidation and consumption of antioxidant enzymes, CAT and GSH-Px, in the present study. Yilmaz *et al.* [30] demonstrated that cisplatin did not cause a lipid peroxidation with lower dose than the present one. The present study indicated that only high dose of cisplatin might cause a lipid peroxidation in liver tissue. However, NO level of liver tissue was similar in our study to Yilmaz *et al.* that cisplatin injection caused high NO level in liver tissue. This NO production might be due to activation of inducible nitric oxide synthase (iNOS) which has role during inflammatory process. The treatment with erdosteine ameliorated the cisplatin-induced liver injury. The CAT, GSH-Px and SOD activities were increased with erdosteine treatment against cisplatin in this study. Meanwhile, the elevated enzyme activities of SOD, CAT and GSH-Px in the cisplatin plus erdosteine group indirectly showed an increase in the number of free radicals after cisplatin administration, and also reflected that these enzymes played important roles in clearing away excessive free radical. Naziroglu *et al.* indicated that antioxidant treatment with selenium and high dose vitamin E against cisplatin caused high antioxidant enzyme activities [6]. In a similar manner, cisplatin-induced high NO level was decreased by erdosteine treatment that might be due to decrease in iNOS activity. Fadillioglu *et al.* demonstrated that erdosteine prevents doxorubicin induced high NO production in myocardial tissue [31].Under aerobic conditions, NO reacts with superoxide anion and forms a radical, peroxynitrite. Peroxynitrite oxides cellular structures and causes lipid peroxidation. Yildirim *et al.* showed marked elevation in NO level in damaged kidney tissue of the cisplatin-treated rats and erdosteine significantly attenuated this increase [13]. Cisplatin has a potential toxicity against liver tissue as seen in the present study. Erdosteine is a potent protective agent against cisplatin-induced liver injury.

In the light of biochemical and microscopic results, it was concluded that cisplatin-induced liver injury in high dose administration and oral erdosteine treatment prevented this toxic side effect by the way of its antioxidant and radical scavenging effects. However, we need further investigation to demonstrate the exact mechanism of erdosteine on cisplatin-induced hepatotoxicity.

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