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OTOLOGY

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The protective effect of erdosteine against ototoxicity induced by cisplatin in rats

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Abstract The elimination of cisplatin ototoxicity is an ongoing concern. This experimental study was undertaken to investigate the effect of oral erdosteine in ameliorating cisplatin-induced ototoxicity. Twenty-eight adult female Wistar albino rats were randomly divided into four equal groups. Group A received an oral carrier vehicle of the drug erdosteine with 0.2 ml of 0.9% saline. Group B was administered only erdosteine (per oral 10 mg/kg twice a day) for 6 days. Group C was injected with cisplatin intraperitoneally (i.p.) on day 0 (16 mg/kg body weight), once only. Group D was given erdosteine (per oral 10 mg/kg/day) 1 day before and for 5 days consecutively after cisplatin injection (16 mg/kg, i.p.). Distortion product otoacoustic emissions (DPOAEs) were elicited in different frequency regions, ranging from 1,001 to 6,299 Hz as DPgram and input/output (I/O) functions from the control and experimental animals. All experimental animals were killed under general anesthesia on day 5, following the last otoacoustic emission measurements. Prior to death, blood samples were drawn for measurement of superoxide dismutase, xanthine oxidase (XO), malondialdehyde and nitric oxide. Initial DPgram and I/O function baseline measurements were similar in all groups prior to any drug administration ($P > 0.05$). On day 5, intra-subject measurement parameters of DPgrams and I/O functions in the cisplatin group showed significant deterioration ($P < 0.05$). The other groups revealed no differences between their pre- and post-test drug administration DPgrams and I/O functions at any test frequency ($P > 0.05$). Comparison of the amplitudes of DPgrams and I/O functions between the cisplatin and control groups showed significant changes ($P < 0.05$). Biochemical studies noted an increased XO activity following cisplatin administration ($P < 0.007$). The other biochemical results did not show significant differences between the study and control groups. This study demonstrates that, in rats, erdosteine is protective for cochlear function against the disruptive effects of cisplatin as measured by DPOAEs.

Keywords Ototoxicity · Cisplatin · Antioxidants · Erdosteine · Otoacoustic emissions · Reactive oxygen species · Xanthine oxidase · Superoxide dismutase · Malondialdehyde · Nitric oxide

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Introduction

Cisplatin, containing the heavy metal platinum, is an effective therapeutic agent for a number of human cancers, including carcinoma of the head and neck, testicle, ovary, bladder, uterus and cervix, bone and lung. Although widely used clinically, the associated side effects of ototoxicity and nephrotoxicity often limit its dosage. Intravenous hydration and diuresis effectively decrease the severity of nephrotoxicity. However, ototoxicity still poses a major limitation to effective cisplatin chemotherapy [2, 9, 25]. Following the administration of high doses of cisplatin, patients frequently have bilateral, irreversible severe to profound sensorineural hearing loss and tinnitus [12]. While the mechanism of cisplatin ototoxicity is still poorly understood, the generation of

reactive oxygen species (ROS), leading to cellular damage in the cochlea, probably plays a significant role [6, 19]. The use of agents to prevent the effects of ROS has been reported [4, 5].

ROS are formed by several mechanisms in physiological conditions, the xanthine oxidase (XO) pathway in purine catabolism being an important one. Cells have developed several defense mechanisms against oxidative attacks, including free radical scavenger enzymes and the utilization of reducing substances, such as vitamin C, tocopherols, glutathione, D-methionine, sodium thiosulfate and carotene [24, 30]. Superoxide dismutase (SOD) participates in free radical metabolism, catalyzing the conversion of the toxic superoxide radical to hydrogen peroxide. The level of activity of this enzyme may reflect the extent of cellular exposure and potential. The primary targets of ROS attack are the polyunsaturated fatty acids in the membrane lipids. The resultant lipid peroxidation may lead to cellular disorganization and yield a wide variety of end products, including malondialdehyde (MDA) [29]. Nitric oxide (NO), a water- and lipid-soluble free radical generated from L-arginine by the action of nitric oxide syntheses, plays an important role in modulating tissue injury in normal and in several pathological conditions.

Erdosteine [N-(carboxymethylthioacetyl)-homocysteine thiolactone] is a thiol derivate mucoactive drug recently introduced into clinical practice [8]. Its molecule contains two sulfur atoms, one of which is present in the aliphatic side chain and the other enclosed in the heterocyclic ring [3]. Erdosteine itself does not have a free thiol group, but its active hepatic metabolites contain a sulphydryl group [1]. The reducing potential of these sulphydryl groups accounts for the free radical scavenging and antioxidant properties of erdosteine [15].

The aim of this study was to investigate the potential protective effect of erdosteine on cisplatin ototoxicity using otoacoustic emissions (OAEs) and some oxidant and antioxidant biochemical parameters in plasma.

Materials and methods

Chemicals

Cisplatin (cisplatinum ebewe 0.5 mg/ml) and erdosteine were obtained from the Liba Drug Company, Turkey, and Ilsan Drug Company, Turkey, respectively. All chemicals were of the highest quality commercially available.

Animals

Twenty-eight adult female Wistar albino rats, weighing between 150 and 240 g at 12 weeks, were used in this study. The animals were fed a standard commercial diet and given water ad libitum during the experimental period. They were housed in a room at 20–22°C ambient temperature with a relative humidity of 50 ± 5% and 12:12 h of light and dark cycle. Plastic cages containing wood-chip bedding each housed three to four rats. The presence of a Preyer's reflex was used for the initial screening of the animals. A normal examination of the outer ear canals and tympanic membranes and normal distortion product otoacoustic emissions (DPOAEs) confirmed the health of the hearing of the adult rats. All animals were given 1 week to acclimatize before the experiment. This research was performed under an animal use protocol approved by the local institutional Animal Care and Use Committee.

Anesthesia

The rats were anesthetized with a cocktail of ketamine hydrochloride (30 mg/kg) and xylazine (6 mg/kg) administered intraperitoneally (i.p.) before the OAEs recordings and sacrifice procedures.

Experimental design

The animals were randomly divided into four groups of seven rats each (group A, control; group B, erdosteine; group C, cisplatin; group D, cisplatin plus erdosteine). Group A received the carrier media vehicle orally for the drug erdosteine with a 0.2-ml volume of 0.9% saline. Group B was administered only erdosteine (per oral 10 mg/kg twice a day) for 6 days. Group C was injected with cisplatin i.p. only on day 0 (16 mg/kg body weight). Group D received erdosteine (per oral 10 mg/kg twice a day by a disposable plastic syringe) 24 and 12 h before and at the same time as the single-dose cisplatin injection (16 mg/kg i.p.) and every 12 h for 5 consecutive days. In all groups, baseline DPOAE testing preceded the administration of the drugs. Body weights, clinical signs and food and water consumption were recorded regularly. The animals were tested before and 5 days after cisplatin treatment with or without chemo protection. The animals in all groups were killed under anesthesia on day 5 following the last OAEs measurements. Before sacrifice, inferior vena cava blood samples were drawn into commercial tubes containing EDTA after centrifugation; the plasma was stored at −30°C until analysis. Experiments were performed between 4.30 and 12:00 p.m. every study day.

OAE measurements

Only rats with normal replicable OAEs before the administration of any substance on day 0 were included in this study. OAEs recordings were performed in a quiet room. The DPOAEs were elicited from the right ear of control and experimental animals utilizing a standard commercial ILO-96 OAE apparatus cochlear emission analyzer (Otodynamics Ltd., London, UK). Following

anesthesia, the primary tones were introduced into the animals' outer ear canal through an insert earphone, using a plastic adapter that sealed the probe in the outer ear canal.

For DPOAE measurements, the intensities of primary stimuli were set as equilevel (L1 = L2) at 65 dB. The frequencies (f_1 and f_2) were adjusted in such a manner that $f_2/f_1 = 1.21$. DPOAEs were determined as DPgrams. The intensity levels of the primary tones were held constant, and DPOAE data were recorded for different frequency regions, ranging from 1,001 to 6,299 Hz, and plotted as a function of f_2. The resolution of the DPgram was obtained at four points per octave. Detection threshold and suprathreshold measures in the form of I/O functions were obtained by decreasing the primary tones from 75 to 36 dB SPL in 3-dB steps. The level of the noise floor was measured at a frequency 50 Hz above the DPOAE frequency, using similar averaging techniques. An emitted response was accepted if the DPOAE at $2f_1$-f_2 amplitude was ≥3 dB above the magnitude of the noise-floor level at the $2f_1$-f_2 + 50 Hz frequency for the DPgram and I/O functions. Both types of testing methods were recorded until the response attained its highest level and then was terminated; no increase was noted.

The baseline hearing status of all animals was determined with DPgram and I/O function measurements. Measurements were made in all animals during experiments on day 0 and 5. For each animal, I/O functions at 3, 4, 5 and 6 kHz frequencies were recorded. The f_2 frequencies examined for the DPgram ranged from 1,001 to 6,299 Hz (1,001, 1,184, 1,416, 1,685, 2,002, 2,380, 2,832, 3,369, 4,004, 4,761, 5,652 and 6,299 Hz.). I/O functions and thresholds were calculated for each group of subjects. After the animals were killed, the disappearance of the DPOAEs of the rats was observed for the purpose of demonstrating the validity of measurements.

Biochemical determinations

After blood samples were centrifuged at 1,500× *g* for 10 min (at +4°C), plasma samples were separated and stored frozen at −30°C. The plasma MDA level was determined by a method based on the reaction with thiobarbituric acid (TBA) at 90–100°C [10]. In the TBA test reaction, MDA or MDA-like substances and TBA react together for 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 (Ultra spec Plus, Pharmacia LKB Biochrom Ltd., England). The results were expressed as micromoles per liter plasma (μmol/l) according to a standard graphic, prepared with serial dilutions of standard 1,1,3,3-tetramethoxypropane.

Since NO is a very labile molecule, its direct measurement in the biological samples is very difficult. In aqueous solution, NO reacts with molecular oxygen and accumulates in the plasma as nitrite (NO_2^-) and nitrate (NO_3^-) ions. These stable oxidation end products can be readily measured in biological fluids and have been used in vitro and in vivo as indicators of NO production [17]. For total nitrite detection, deproteinized plasma was treated with copperized cadmium (Cd) granules to reduce NO_3^- to NO_2^-. Nitrite concentrations were quantified by a colorimetric assay based on the Griess reaction [7]. Briefly, a chromophore with a strong absorbance at 545 nm is formed by the reaction of nitrite with a mixture of N-naphthylethylene diamine and sulphanilamide. A standard curve was established with a set of serial dilutions (10^{-8} to 10^{-3} mol/l) of sodium nitrite. Results were expressed as micromole per liter plasma (μmol/l).

The principle of the total SOD (EC 1.15.1.1) activity method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system [27]. Activity was assessed in the ethanol phase of the plasma after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of the plasma and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per milliliter plasma (U/ml).

Plasma XO (EC 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm [22]. A calibration curve was constructed by using 10–50 milli-units/ml concentrations of standard XO solutions (Sigma X-1875). One unit of activity was defined as 1 μmol uric acid formed per minute at 37°C, pH 7.5. Results were expressed in units per liter of plasma.

Statistical analysis

The non-parametric Kolmogorov-Smirnov test was used to determine the normality of the distribution of the OAE values. The normality hypothesis was rejected for all of the variables. Since all variables did not show a normal distribution, the Kruskall-Wallis test was used to compare the median values of the OAE data among four study groups to determine the differences in amplitudes of DPOAEs and corresponding noise floor differences and thresholds for each frequency. Additionally, the Wilcoxon signed rank test was used to compare the OAE values of each group obtained during the baseline measurements and those obtained during the 5th day of the study. Biochemical data were analyzed using the Mann Whitney U test, and weight loss data were analyzed statistically by the one-way ANOVA test.

Differences were considered significant ($P < 0.05$) in all the analyses. Data were analyzed by using SPSS (10.0 version).

Results

The animal subjects tolerated the anesthesia well. There were no general health differences among subjects that received the carrier vehicle or erdosteine alone (groups A and B). However, the animals being injected with cisplatin alone (group C) and cisplatin plus erdosteine (group D) showed significant weight loss on day 5 compared to other groups (Fig. 1). In addition, food and water consumption decreased in this group day by day. One rat was lost due to systemic toxicity before the 5th day of OAE recordings in the cisplatin group.

Hearing studies with OAE

DPgram and I/O functions of study groups corresponding to days 0 and 5 are presented in Figs. 2, 3, 4 and 5. On day 0, the initial baseline DPOAE measurement results presented comparable values in all groups prior to drug administration ($P > 0.05$). On day 5, intrasubject measurement parameters of the DPgrams and I/O functions of the cisplatin group were found to have deteriorated significantly ($P < 0.05$). The second measurements of the other groups revealed no significant differences between their DPgrams and I/O functions at all frequencies ($P > 0.05$).

In DPgrams, the emission amplitude levels were greater than the noise floor throughout the testing frequencies for all sessions. The analyses of the results as the median amplitudes of DPgrams and I/O functions revealed statistically significant differences between groups C and D ($P < 0.05$), suggesting that erdosteine had a protective effect against cisplatin ototoxicity.

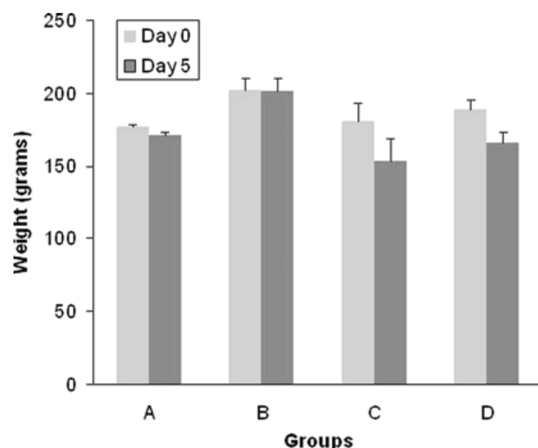

Fig. 1 Weight means of the rats on days 0 and 5. Control group with saline (**A**); control group with erdosteine only (**B**); rats treated with cisplatin (**C**); rats treated with cisplatin plus erdosteine (**D**). *Error bars* are expressed as the SEM

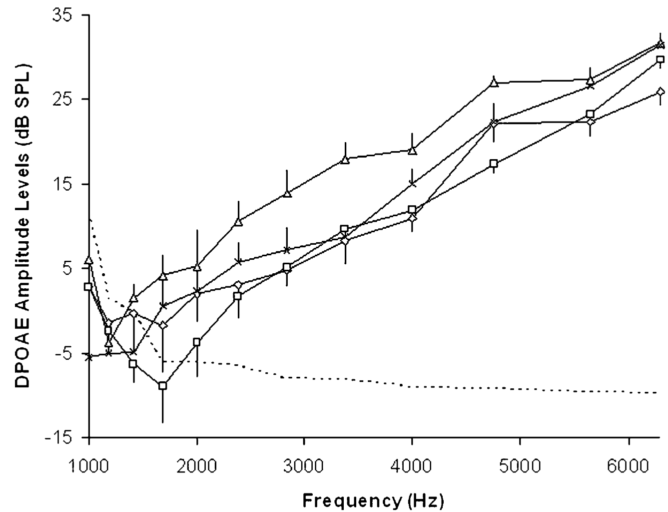

Fig. 2 DPgrams measured from four groups on day 0. Control group with saline (◇); control group with erdosteine only (□); rats treated with cisplatin (Δ); rats treated with cisplatin plus erdosteine (x). The *dotted line* is the noise level. *Error bars* are expressed as the SEM

Biochemical studies of plasma

The results of the biochemical studies of plasma are presented in Fig. 6. The administration of erdosteine alone did not affect any of the parameters studied. Plasma XO activity was found to be increased in cisplatin-administrated rats ($P < 0.007$). However, erdosteine administration prevented that increase in XO activity.

Discussion

Cisplatin has a strong antitumor activity against several tumors, including head and neck squamous cell carcinoma. However, severe side effects, including ototoxicity, remain; cisplatin impairs cochlear function in experimental animals, causing hearing loss and loss of outer hair cells [4]. Although there are several reports indicating the involvement of destructive free radical metabolism in cisplatin-induced ototoxicity, the mechanism underlying the problem has not been completely clarified as yet. The mechanism of cisplatin ototoxicity is based on the generation of the ROS, which interfere with the antioxidant protection of the organ of Corti [18, 23]. ROS reacts with cell membrane lipids to produce toxic aldehydes such as 4-hydroxynonenal. These are implicated in apoptosis and cell death in the organ of Corti explants and spiral ganglion cell cultures [14].

The search for ototoxicity was assessed 5 days after cisplatin administration to reveal the short-term ototoxic effects of cisplatin. Cisplatin was injected in a

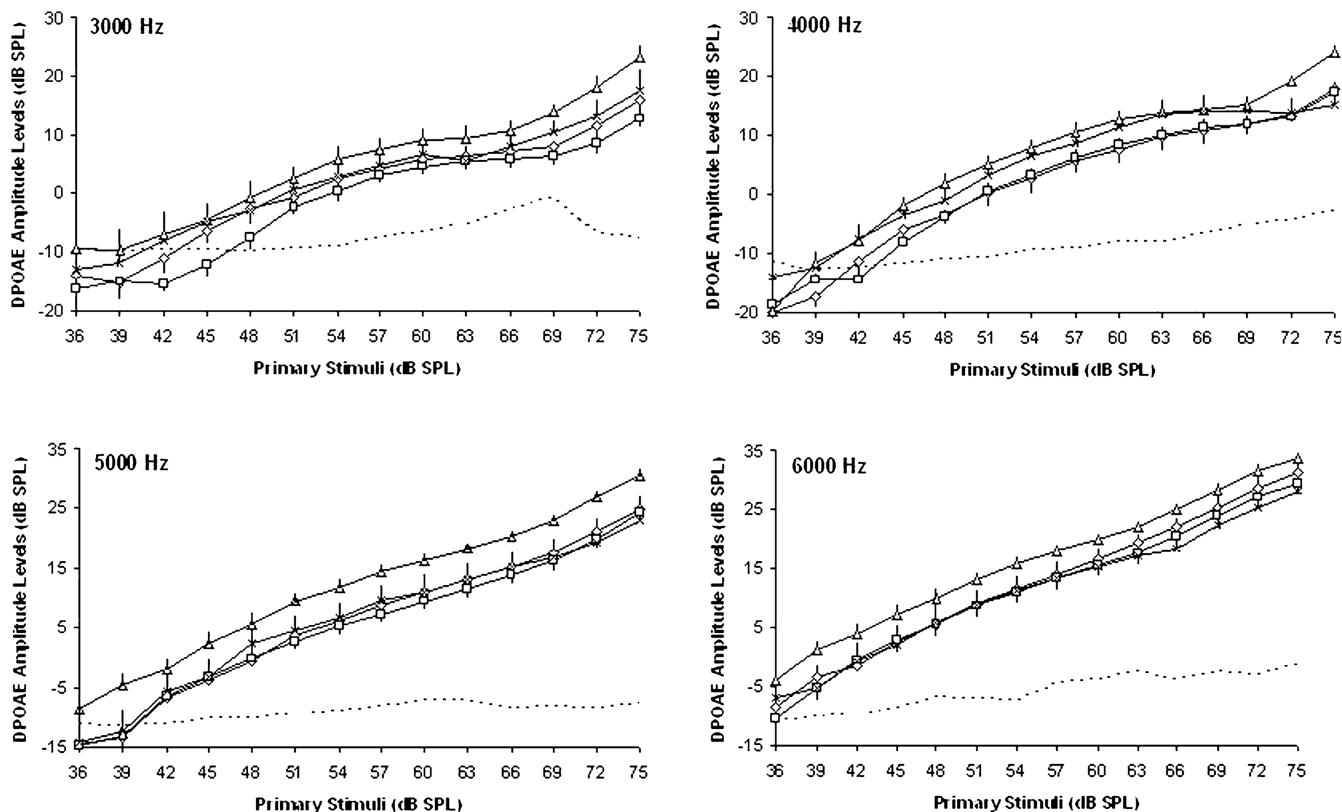

Fig. 3 I/O functions of the DPOAEs at 3, 4, 5 and 6 kHz frequencies of four groups on day 0. Control group with saline (◇); control group with erdosteine only (□); rats treated with cisplatin (Δ); rats treated with cisplatin plus erdosteine (x). The *dotted line* is the noise level. *Error bars* are expressed as the SEM

single high dose, because in most oncology clinics, high doses of cisplatin are usually administered once every 3 or 4 weeks. We monitored hearing loss using DPOAEs, which are highly selective for the cochlea. The most important benefits of OAEs are their non-invasive capacity and objectivity to determine the early stages of sound processing and evaluate the biomechanical activity of the outer hair cells [19]. DPOAE measurement is a well-described method for detecting the effects of cisplatin on the cochlea before changes are detected by pure-tone audiometry [21].

Erdosteine is a mucolytic and mucoregulator agent that modulates mucus production and viscosity and increases mucociliary transport [20]. The beneficial effects of erdosteine in the prevention of ROS complications in the rat and human lung have been reported [1, 13, 28]. Recently, Yildirim et al. [31] showed that erdosteine may be a promising drug for protection against cisplatin-induced nephrotoxicity. The evidence in the present study indicates that erdosteine could effectively protect the cochlea by its antioxidant effect from cisplatin-induced ototoxicity. Erdosteine also significantly prevented the increase of XO activity. Fadillioglu and Erdogan [11] suggested that doxorubicin administration results in increased lipid peroxidation in plasma as well as erythrocytes. Erdosteine treatment helped to prevent oxidative injury by increasing antioxidant enzymes, especially SOD in rats. On the contrary, we did not find any differences in plasma SOD activity in the study groups. SOD is an antioxidant enzyme that aids in the detoxification process of superoxide radicals. Although it is well known that the oxidative process, including SOD, plays a role in cisplatin ototoxicity [5], we did not observe a significant change in the activity of SOD.

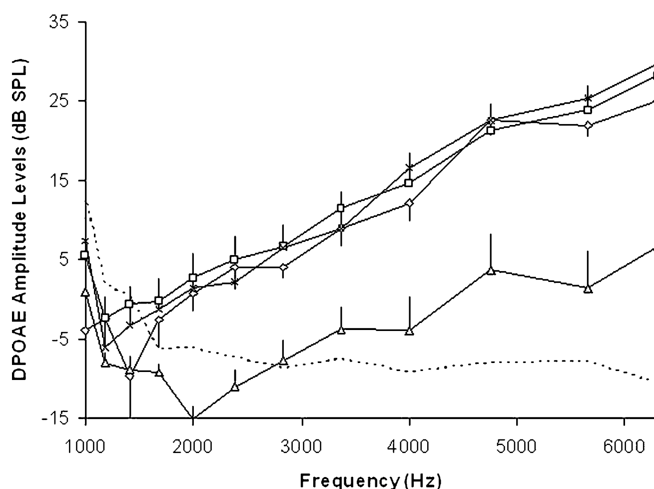

Fig. 4 DPgrams measured for four groups on day 5. Saline (◇); erdosteine (□); rats treated with cisplatin (Δ); rats treated with cisplatin plus erdosteine (x). The *dotted line* is the noise level. *Error bars* are expressed as the SEM

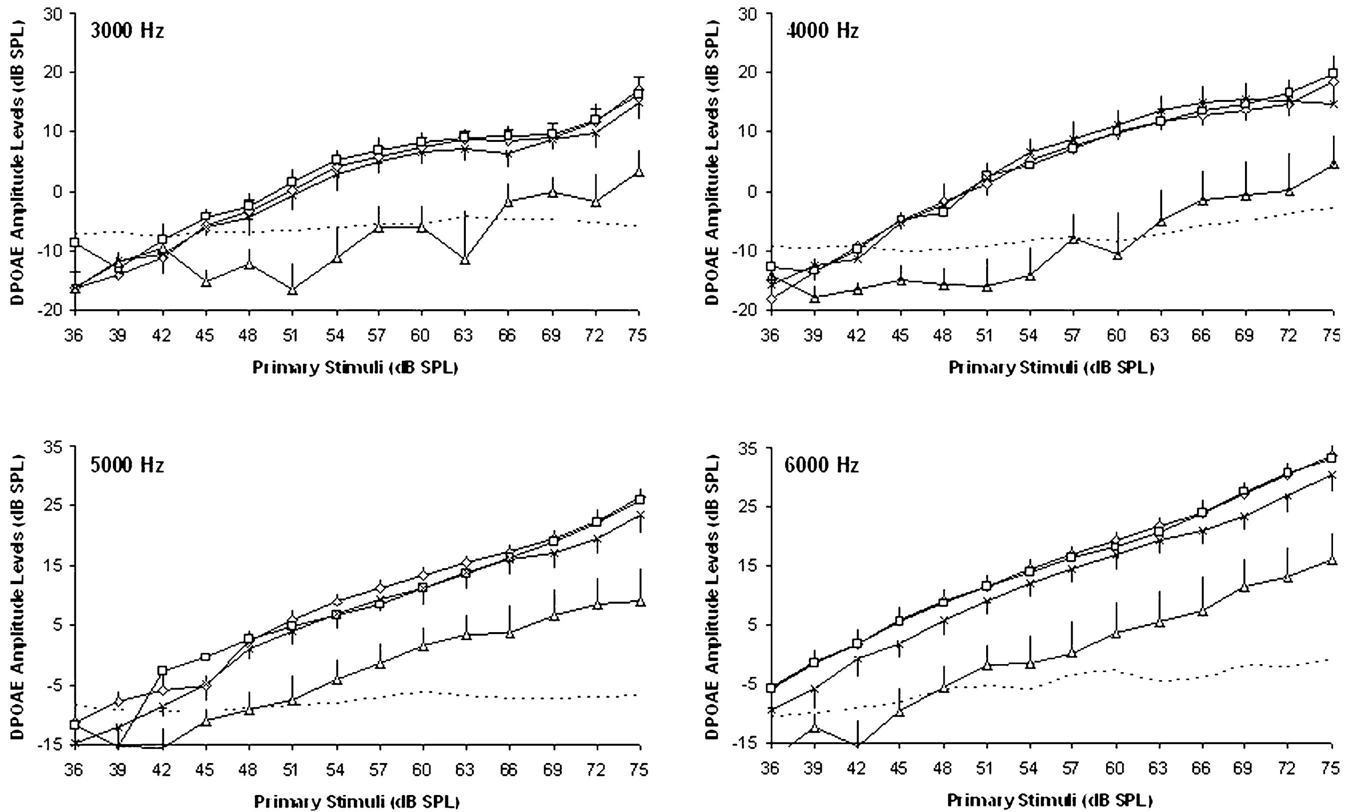

Fig. 5 I/O functions of the DPOAEs at 3, 4, 5 and 6 kHz frequencies of four groups on day 5. Control group with saline (◇); control group with erdosteine only (□); rats treated with cisplatin (Δ); rats treated with cisplatin plus erdosteine (x). The *dotted line* is the noise level. *Error bars* are expressed as the SEM

Failure to detect change in MDA and NO levels points to a limited role of increased oxidative stress and diminished enzymatic antioxidants in cisplatin-induced ototoxicity. The fact that NO may react with superoxide radicals to produce peroxynitrite in in-vivo conditions may be the main reason for the lack of change in plasma NO levels in different study groups of rats.

Several factors may affect the plasma enzyme activity level, including leakage from cells and altered production. Therefore, all the changes in the enzyme activities in the cellular compartment may contribute to the levels and/or activities of the same enzymes in plasma. Thus, the change in XO enzyme activity in plasma may be a measure of the changes in tissues and organs as well [26]. On the other hand, ATP catabolism results in the local release of its metabolite adenosine. This can be further deaminated to inosine by the action of adenosine deaminase. Inosine is further metabolized to uric acid by XO. A burst of XO-mediated free radical generation in tissues is triggered by a large increase in substrate formation, as seen with the degradation of adenine nucleotides. In the present study, we found that cisplatin application increased XO activity. But this finding does not substantiate that ATP degradation occurred. XO exists primarily as the xanthine dehydrogenase (desulfo) form and can be converted to XO (D-to-O conversion) by a variety of conditions, including ischemia-reperfusion, proteolysis, homogenization, sulphydryl oxidation, storage at −20°C and anaerobiosis [16]. Cisplatin alone or some factors mentioned above may result in XO elevation, possibly from xanthine dehydrogenase to XO conversion in plasma. The in vivo or in vitro effects of cisplatin on XO activity or on xanthine dehydrogenase-XO conversion have not been investigated as yet.

The other interesting finding in this experimental study was the diminishing effect of erdosteine on XO activity. It can be speculated that erdosteine may donate its reducing equivalents of sulphydryl groups to the XO enzyme and regenerate xanthine dehydrogenase again. Therefore, XO activity may decrease and xanthine dehydrogenase activity may increase gradually upon erdosteine administration to the rats during cisplatin toxicity. Because we did not measure xanthine dehydrogenase activity in plasma, we can only suggest this as a possibility.

The findings of this study have been restricted by the frequency spectrum of the commercially available DPOAE instrument designed for human use. Higher frequency measurements might be able to reveal more comprehensive information about the protective role of erdosteine against cisplatin-induced ototoxicity.

The study period could be extended in experimental animals to enable the evaluation of the long-term effects, such as the reversibility of ototoxicity as has been

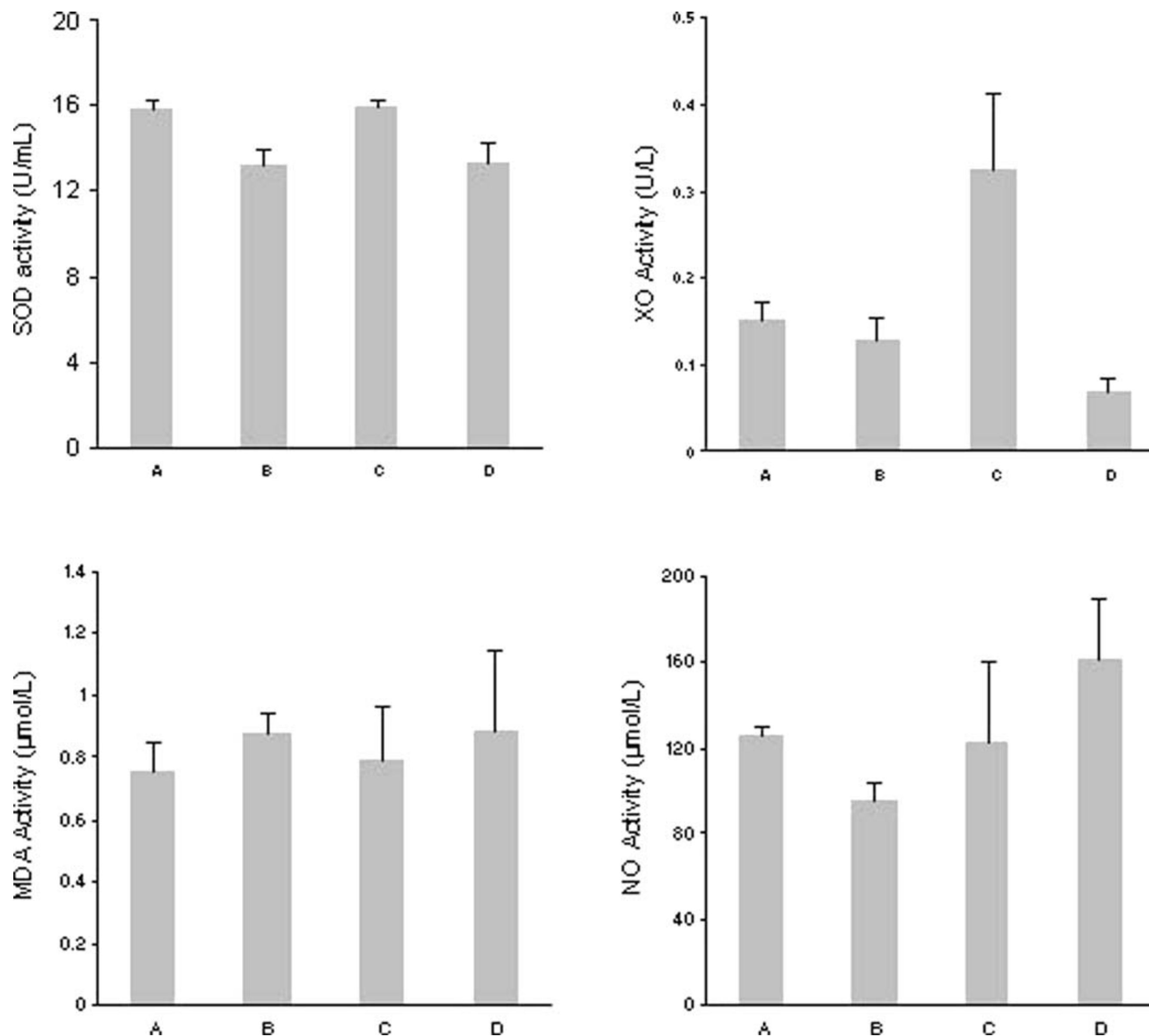

Fig. 6 Superoxide dismutase (*SOD*), xanthine oxidase (*XO*), malondialdehyde (*MDA*) and nitric oxide (*NO*) levels in plasma of the rats belonging to four groups. Saline (**A**); erdosteine (**B**); rats treated with cisplatin (**C**); rats treated with cisplatin plus erdosteine (**D**). *Error bars* are expressed as SEM

reported [19], but high-dose cisplatin injection caused a high mortality rate in rats 4–5 days later. Thus, we planned to determine the short-term effect of cisplatin ototoxicity and attempted to eliminate these effects with erdosteine. The current experiments suggest that erdosteine is a potential candidate drug to eliminate cisplatin-induced ototoxicity, at least at the outer hair cell level.

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