

Aminoguanidine Prevents Ototoxicity Induced by Cisplatin in Rats

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Abstract. Cisplatin (CDDP) is one of the most potent antineoplastic drugs, but its therapeutic use is limited by side effects such as ototoxicity. This study tested the effect of aminoguanidine (AG), a specific inhibitor of inducible nitric oxide synthase, on CDDP ototoxicity. Female Wistar albino rats were randomly assigned to 4 groups: saline controls (n = 7), CDDP (n = 7), CDDP plus AG (n = 7), and AG (n = 7). Rats in the CDDP group received a single injection of cisplatin (16 mg/kg, ip). Rats in the CDDP plus AG group received aminoguanidine (20 mg/kg, ip) twice daily on the day before and on 5 consecutive days after a single injection of CDDP (16 mg/kg, ip). Rats in the AG group received aminoguanidine (20 mg/kg, ip) twice daily for 6 days. Distortion product otoacoustic emissions (DPOAEs) were elicited from the control and experimental animals utilizing a standard commercial otoacoustic emissions apparatus. DPOAEs were measured in the rats on day 0, prior to any drug administration, and on day 5. The initial baseline distortion product diagrams (DPgram) and input/output (I/O) function measurements gave similar results in all 4 groups. On day 5, there was significant deterioration of the DPgrams and I/O functions in the CDDP group; no significant changes of DPgrams and I/O functions were observed on day 5 in the other 3 groups. The median amplitudes of DPgrams and I/O functions revealed significant differences between the CDDP group and the other 3 groups. These results suggest that AG had a preventive effect against CDDP ototoxicity. In summary, this study indicates that AG prevents the cochlear dysfunction and hearing loss induced in rats by a single dose of CDDP. (received 6 February 2005; accepted 18 April 2005)

Keywords: ototoxicity, cisplatin, aminoguanidine, otoacoustic emissions

Introduction

Cisplatin (cis-diaminedichloroplatinum [II], CDDP) is widely used as a chemotherapeutic agent for several human cancers, including head-neck, testicular, ovarian, bladder, uterine cervical, osteogenic, and lung carcinomas. However, CDDP administration is often accompanied by side effects that have limited the dosage and duration of CDDP treatment. The main toxicities include ototoxicity, nephrotoxicity, gastrointestinal toxicity, neurotoxicity, and myelosuppression. The primary dose-

limiting side effects of CDDP are nephrotoxicity and bilateral irreversible severe sensorineural hearing loss and tinnitus [1,2]. Although use of intravenous hydration and diuresis effectively decreases the severity of nephrotoxicity, ototoxicity still poses the major limitation to effective doses of CDDP chemotherapy [3,4]. Mechanisms of CDDP ototoxicity are not fully understood, but reactive oxygen species (ROS) have been implicated in its pathogenesis. In rats with documented CDDP ototoxicity, loss of inner and outer hair cells, degeneration of the stria vascularis, and significant decrease in the number of spiral ganglion cells have been reported [5].

Nitric oxide synthase (NOS) is the enzyme that produces nitric oxide (NO) from L-arginine in cells. Three isoforms of NOS have been identified in mammals: (i) the neuronal type (nNOS), (ii) the

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endothelial type (eNOS), and (iii) the inducible type (iNOS) [6]. nNOS and eNOS are expressed constitutively in neurons and endothelial cells among other cell types. NO production is dependent on Ca^{2+} /CaM binding [7] and has a wide distribution in the cochlear cells [8,9]. nNOS may play a role in hair cell physiology [10] and eNOS is involved in the regulation of cochlear blood flow [11]. iNOS activity is controlled at the transcriptional level and that isoform produces NO at a high rate. Effects of NO are imperative for physiological functions but overproduction of NO is harmful for tissues because NO is a free radical. Fortunately, iNOS is not activated under normal physiological conditions. Reactive oxygen species (ROS) induce lipid peroxidation that damages various tissues, including the structures of the cochlea; ROS been suggested to be responsible for CDDP ototoxicity [12,13]. There is a great interest in preventing the effects of ROS and thereby reducing the toxicity of CDDP. Thus, various agents (eg, antioxidants) have been administered in combination with CDDP and appear to ameliorate its side effects, including ototoxicity [13-16].

Aminoguanidine (AG) is a relatively specific inhibitor of iNOS [17]. Kelly et al [18] investigated the effects of AG (50 mg/kg, twice daily) on CDDP-induced ototoxicity in rats. By monitoring auditory brainstem evoked responses (ABR), they showed that AG ameliorates CDDP-induced ototoxicity. They suggested that AG acts directly to scavenge hydroxyl radicals. In this study we used otoacoustic emission (OAE) measurements to test the protective effect of a lower dosage of AG on ototoxicity induced by CDDP.

Materials and Methods

Chemicals. Cisplatin (cisplatinum, Ebewe, 0.5 mg/ml) was obtained from Liba Drug Company, Turkey. Aminoguanidine was purchased from SIGMA, Germany. Other chemicals were of the highest quality commercially available.

Animals. Female Wistar albino rats ($n = 32$, initial weight = 170 to 225 g, age = 12 wk) were used in this study. The animals were fed standard rat chow and drinking water ad libitum. They were kept in plastic cages with wood-chip bedding (3-4 rats/cage) in a room with ambient temperature 20-22°C, relative humidity $50 \pm 5\%$, and 12:12 hr light/dark cycle. The

presence of Preyer's reflex was used for acceptance of each rat to the study. The ear canals and tympanic membranes of the rats were examined initially by otomicroscopy. In addition, distortion product otoacoustic emissions (DPOAEs) testing confirmed that their hearing was normal and that they were suitable for the investigation. All animals were observed for one week before the experiments. The experimental protocol was approved by our Institutional Animal Care and Use Committee.

Anesthesia. Rats were anesthetized with ketamine hydrochloride (30 mg/kg) and xylazine (6 mg/kg), administered ip before the OAEs recordings.

Experimental design. The rats were assigned to 4 groups: (i) untreated control group ($n = 7$), (ii) CDDP group ($n = 11$), (iii) CDDP plus AG group ($n = 7$), and (iv) AG group ($n = 7$). Rats in the CDDP group received a single injection of CDDP (16 mg/kg, ip) at time 0. Rats in the CDDP plus AG group received AG (20 mg/kg, ip) twice daily on the day before and the 5 consecutive days after CDDP injection (16 mg/kg, ip). Rats in the AG group received only AG (20 mg/kg, ip) twice daily for 6 days. Body weights, clinical signs, and food and water consumption were recorded regularly. Hearing tests by OAE measurements were performed in all rats on day 0 (baseline) and on day 5.

OAE measurements. All rats in this study had normal ear canals and tympanic membranes, based upon otomicroscopic examination, and normal replicable OAEs before the administration of any substance on day 0. Repeated OAEs recordings were made on the left ear of each rat. The OAEs recordings were performed in a quiet room. The DPOAEs were elicited by use of a commercial OAEs apparatus (ILO-96 cochlear emission analyzer, Otodynamics, Ltd., London, UK). Following anesthesia, the primary tones produced by 2 separate speakers were introduced into the animal's outer ear canal through an insert earphone probe using a plastic adapter that adapts and seals the probe in the outer ear canal.

DPOAEs were measured as sound pressure level (SPL) using stimuli of constant intensity and frequency changes. For distortion product diagrams (DPgram), the intensities of primary stimuli were set as equilevel ($L_1 = L_2$) at 65 dB. The frequencies (f_1 and f_2) were adjusted so that $f_2/f_1 = 1.21$. Detection thresholds 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 that was 50 Hz above the DPOAEs frequency, using a similar averaging technique. An emitted response was accepted if the DPOAEs at $2f_1-f_2$ amplitude were 3 dB above the magnitude of the noise-floor level at the $2f_1-f_2 + 50$ Hz frequency for DPgram and input/output (I/O) functions. Both types of testing were recorded until the responses were maximal; recording was stopped when further testing did not increase the DPOAEs amplitude levels.

DPOAE measurements were made in all rats on day 0 and on day 5. Otomicroscopic examination of the rats was

performed before every DPOAE test to exclude middle ear pathology that may impair DPOAE measurements. For each rat, I/O functions at 3, 4, 5, and 6 kHz were recorded and the detection threshold was noted. The f2 frequencies examined for DPgram ranged from 1 to 6.3 kHz (1001, 1184, 1416, 1685, 2002, 2380, 2832, 3369, 4004, 4761, 5652, 6299 Hz). Separate threshold and I/O functions were calculated for each group of rats. After each rat was killed, disappearance of the rat's DPOAEs was checked to confirm the validity of the measurements.

Statistics. Each rat served as its own control for OAEs recordings. Results were analyzed by the Kruskal Wallis and Wilcoxon tests (SPSS program, version 10.0) to test for differences in amplitudes of DPOAEs, the corresponding noise floors differences, and the thresholds for each frequency. Effects of AG and CDDP were evaluated for intrasubject and inter-subject variation, looking at baseline measurements and median values; p values <0.05 were considered statistically significant.

Results

The anesthesia was well tolerated by the rats. No adverse effects were observed in rats that received only AG injections twice a day. The rats that received the combination of CDDP and AG showed a mild degree of systemic toxicity. The rats injected with CDDP alone showed the greatest degree of systemic toxicity, with increased weight loss on day 5 compared to other groups ($p < 0.05$), and decreased food and water consumption in this group day-by-day. In the CDDP group, 1 rat died on day 4 and 3 rats died on day 5. There were no deaths in the other 3 groups.

The DPgram and I/O functions of the study groups on day 0 and day 5 are shown in Figs. 1-4. On day 0, prior to any injections, the initial DPOAEs measurement results gave similar values in the 4 groups ($p > 0.05$). On day 5, the intrasubject measurement parameters of DPgrams and I/O functions of the CDDP group were significantly deteriorated ($p < 0.05$). In the other 3 groups, no significant differences were observed for the intrasubject measurement parameters of DPgrams and I/O functions at all frequencies ($p > 0.05$).

In the DPgrams, the emission amplitude levels were greater than the noise floor throughout the testing frequencies on day 0 and day 5. Analysis of the results in the median amplitudes of DPgrams and I/O functions revealed statistically significant

differences between the CDDP group and the CDDP plus AG group ($p < 0.05$), suggesting that AG administration prevented CDDP ototoxicity.

Discussion

CDDP is commonly used for chemotherapy of solid tumors, including germ cell, ovarian, cervical, lung head and neck cancers [19], but has dosage-limiting side effects (eg, ototoxicity, nephrotoxicity). CDDP impairs cochlear function in experimental animals and produces hearing loss and loss of outer hair cells in patients [20,21]. The CDDP ototoxicity evidently derives from the generation of reactive oxygen species that interfere with the antioxidant protection of the organ of Corti [13,22,23]. Reactive oxygen species react with membrane lipids to produce toxic aldehydes, such as 4-hydroxynonenal, which was found to cause apoptosis in organ of Corti explants and in cultures of spiral ganglion cells [13].

NO is an important gaseous material for hearing, constitutively expressed by nNOS and eNOS, and has a wide distribution in the cochlea [8,9,24]. eNOS regulates cochlear blood flow [11,25] and nNOS likely plays a physiological role in hair cells [10]. iNOS is not expressed in the cochlea under normal conditions [8,24], but unregulated NO production by iNOS is associated with various pathological conditions, such as ischaemia-reperfusion injury, septic shock, inflammatory disorders, and adverse effects of drugs such as CDDP [26]. Immunohistochemical studies have shown that iNOS is expressed in the cochlea in conditions such as lipopolysaccharide administration, pneumolysin exposure, noise exposure, endolymphatic hydrops, and gentamicin administration [27,30]. Watanabe et al [31], demonstrated that iNOS immunoreactivity is expressed in the cochlea of CDDP-treated animals. Therefore, NO has a double-edge nature with roles in physiological and pathological conditions.

In this study, AG, a relatively specific iNOS inhibitor [17], was able to ameliorate CDDP-induced ototoxicity in rats in vivo. The beneficial effects of free radical scavenger and antioxidant materials on CDDP-induced hearing loss have been demonstrated [13-16,32-34]. As lipid peroxidation

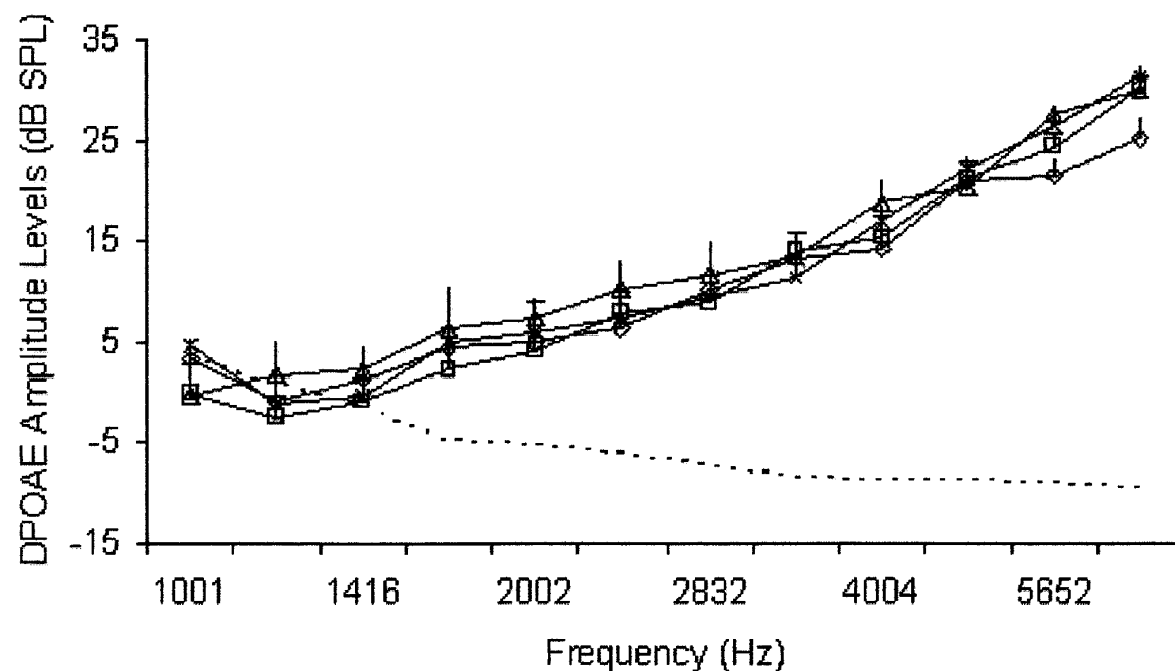


Fig. 1: DPgrams measured from the 4 groups of rats on day 0. Control group with saline (open diamonds); group with AG alone (open squares); group treated with CDDP alone (open triangles); group treated with CDDP plus AG (x). The dotted line is the noise level. The error bars are the SE.

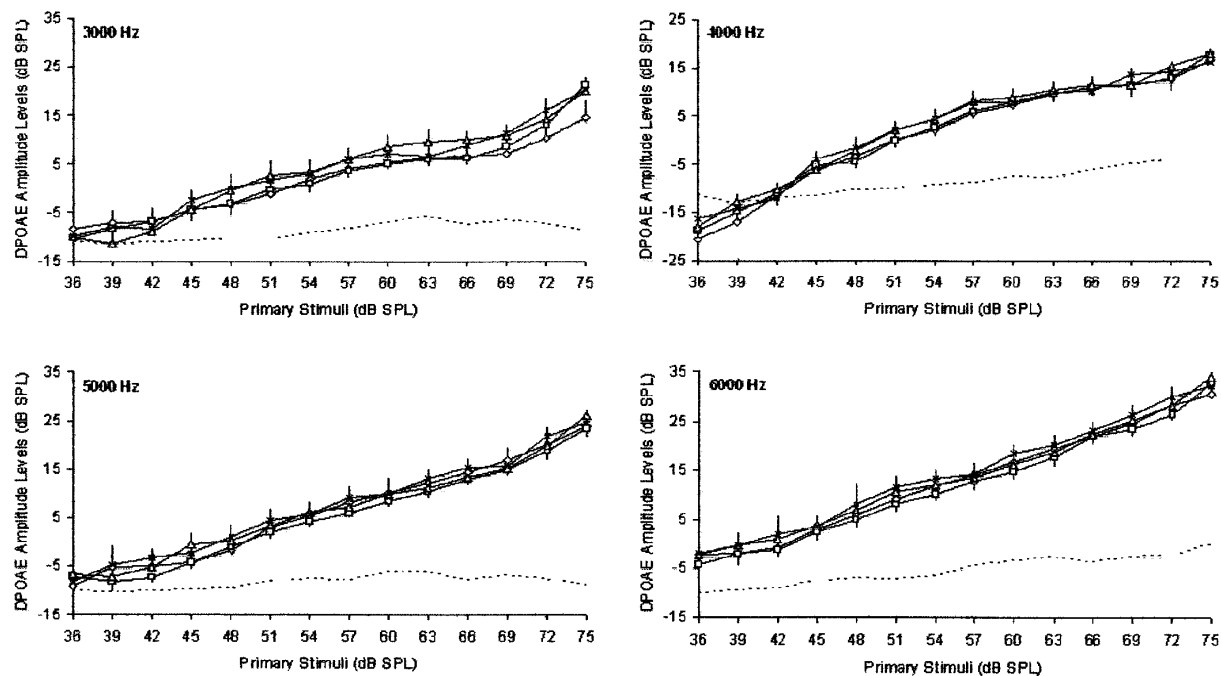


Fig. 2: I/O functions of the DPOAEs at 3, 4, 5, and 6 kHz frequencies in the 4 groups of rats on day 0. Control group with saline (open diamonds); group with AG alone (open squares); group treated with CDDP alone (open triangles); group treated with CDDP plus AG (x). The dotted line is the noise level. The error bars are the SE.

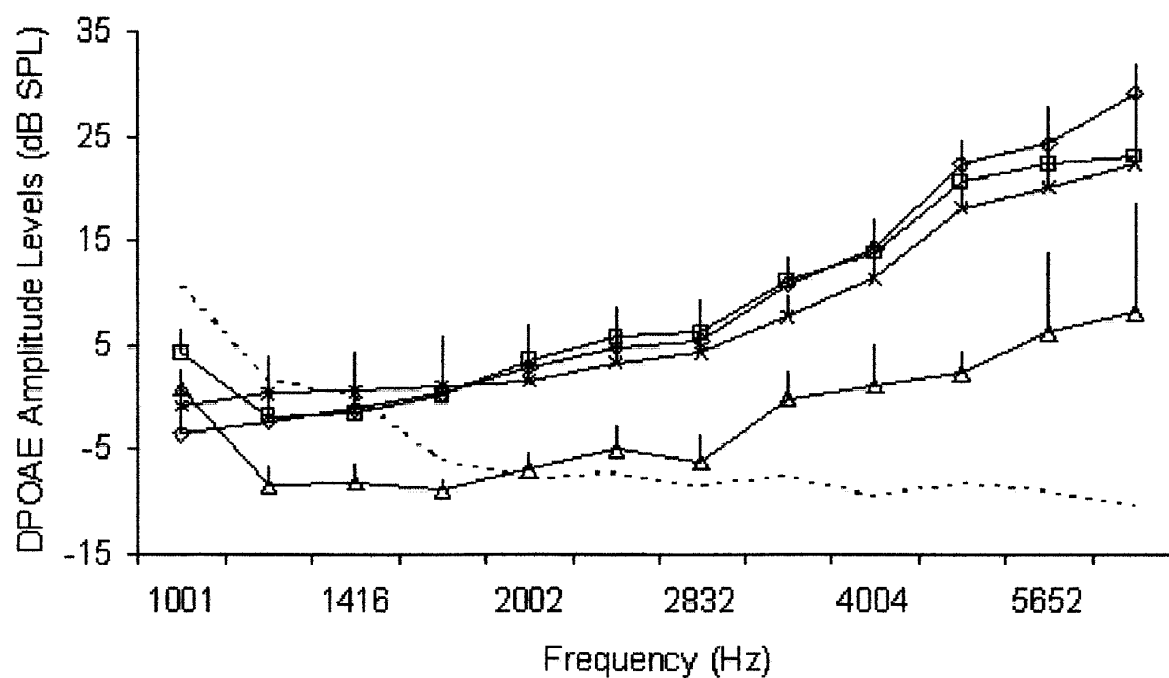


Fig. 3: DPgrams measured in the four groups of rats on day 5. Control group with saline (open diamonds); group with AG alone (open squares); group treated with CDDP alone (open triangles); group treated with CDDP plus AG (x). The dotted line is the noise level. The error bars are the SE.

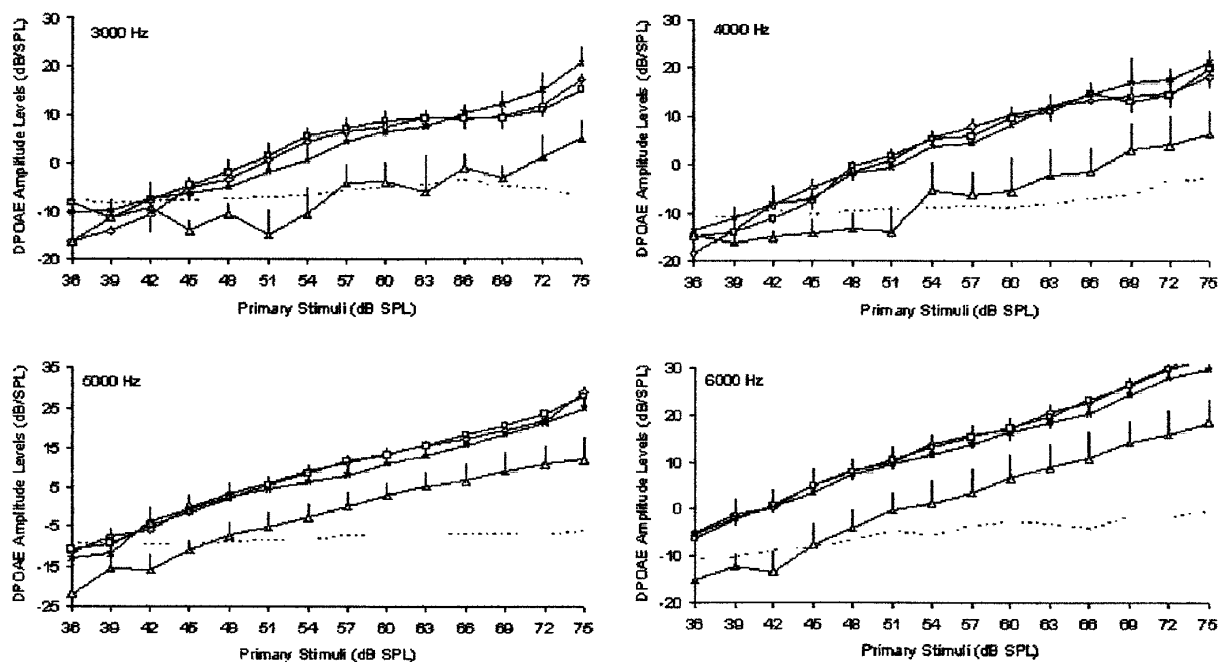


Fig. 4: I/O functions of the DPOAEs at 3, 4, 5, and 6 kHz frequencies in the 4 groups of rats on day 5. Control group with saline (open diamonds); group with AG alone (open squares); group treated with CDDP alone (open triangles); group treated with CDDP plus AG (x). The dotted line is the noise level. The error bars are the SE.

is the main reason for CDDP ototoxicity, prevention of lipid peroxidation appears to be an attractive strategy to protect the cochlea from reactive oxygen-nitrogen species-mediated damage. AG exerts beneficial effects in several conditions including septic shock and ischaemia/reperfusion injury of the kidney and heart by inhibiting iNOS activity [35]. AG also has antioxidant capabilities that could ameliorate free radical-mediated lipid peroxidation and protein modifications by binding aldehydes and ROS [36]. Kelly et al [18] suggested that AG reduces CDDP ototoxicity by directly scavenging hydroxyl radicals. Additionally, Yildirim et al [37] showed that AG significantly prevented depletion of superoxide dismutase and glutathione peroxidase and decreased myeloperoxidase activity, NO, and malondialdehyde levels in lung tissue after bleomycin-induced lung fibrosis. However, Tabuchi et al [38] reported that iNOS inhibition with AG does not protect against ischemia-reperfusion injury of the cochlea. Also, Ruan [39] found that NO/peroxynitrite is unlikely to cause injury in the ischemic cochlea [39].

In the present study ototoxicity was assessed 5 days after CDDP administration to investigate the short-term ototoxic effect of CDDP. We monitored hearing loss using DPOAEs, which is extraordinarily selective for detecting cochlear damage. The most important advantages of OAEs are their non-invasive character, their objectivity to assess the primary stages of sound processing, and their ability to evaluate the biomechanical activity of the outer hair cells [12].

The study period could be extended in experimental animals to assess long-term effects, such as the reversibility of ototoxicity, as reported by other investigators [12,40]. Since high mortality occurs in rats >5 days after CDDP administration at high dosage, the present experimental procedure was designed to assess the short-term manifestations of CDDP ototoxicity and the possible prevention of ototoxicity by administration of AG. The results of the present study suggest that AG is useful in reduction of experimental CDDP ototoxicity in rats. AG may be a potential candidate drug to prevent CDDP-induced ototoxicity at the outer hair cell level in humans.

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