Protection against cisplatin ototoxicity in a Sprague-Dawley rat animal model

Protezione dall'ototossicità indotta da cisplatino nel modello animale di ratto "Sprague-Dawley"

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Key words

Inner ear • Ototoxicity • Cisplatin • D-methionine • Otoacoustic emissions • Auditory Brainstem Responses • Animal model

Parole chiave

Orecchio interno • Ototossicità • Cisplatino • D-metionina • Otoemissioni acustiche • Potenziali evocati uditivi • Modello animale

Summary

Cisplatin (CDDP) is an anti-neoplastic drug extensively used in cases of head and neck cancer. Cisplatin induces numerous untoward side-effects including ototoxicity. In this study, cisplatin ototoxicity in Sprague-Dawley rat animal model has been evaluated and the oto-protection provided by the systemic administration of the antioxidant drug D-methionine has been tested. A total of 12 Sprague-Dawley rats were used: 8 were treated intra-peritoneally with D-methionine (300 mg/kg) and cisplatin (16 mg/kg, slow 30 min-infusion), 4 only with cisplatin. The hearing threshold of the animals was evaluated by electrophysiological procedures as Otoacoustic Emissions and Auditory Brainstem Responses. The effects of protection were evaluated after 72 hours. The data from the Otoacoustic Emissions (in the 4.0-12 kHz band) and Auditory Brainstem Responses recordings suggested that D-methionine can partially protect from Cisplatin ototoxicity.

Riassunto

Il Cisplatino (CDDP) è un farmaco antineoplastico ampiamente utilizzato nel trattamento chemioterapico delle neoplasie della testa e del collo. Il CDDP induce numerosi effetti collaterali, inclusa l'ototossicità. Con il presente studio si è voluta verificare l'ototossicità indotta dal CDDP nel modello animale di ratti "Sprague-Dawley" e quindi anche l'attività di oto-protezione indotta dalla somministrazione sistemica di un farmaco anti-ossidante, quale la D-metionina. A tal fine, sono stati utilizzati 12 ratti "Sprague-Dawley": 8 sono stati trattati con iniezioni intraperitoneali di D-metionina (300 mg/kg) e cisplatino (16 mg/kg, ad infusione lenta di 30 minuti) mentre 4 sono stati trattati solo con CDDP. Le soglie uditive sono state valutate mediante metodiche atte a vagliare la funzione uditiva (emissioni otoacustiche e potenziali evocati uditivi del tronco encefalico); i dati raccolti hanno suggerito che la D-metionina può avere un'azione parzialmente oto-protettiva rispetto all'ototossicità indotta da CDDP.

Introduction

Cisplatin (CDDP) is a widely used anti-neoplastic drug presenting numerous side-effects such as: nausea and vomiting, neurotoxicity [1 2], nephrotoxicity [3], vestibulotoxicity [3] and ototoxicity [4-6]. The latter has been reported in many studies and is mainly characterized by an initial threshold shift at the higher frequencies (i.e., 4.0 and 8.0 kHz) [7]. Frequent and continuous CDDP administration can affect the lower frequencies resulting in a progressive hearing loss and difficulties in speech recognition in noisy environments.

The ototoxic effects of cisplatin have been primarily evaluated in various laboratory animals [8]. The first ototoxic impact seems to involve the cochlea, leading to anatomical changes on the organ of Corti and of the *stria vascularis* [9]. In such cases, it is possible to observe damage in the outer hair cells starting from the first row of the basal turn, alterations of the supporting cells and Reissner's membrane [10]. These morphological alterations are considered as the direct effect of blocking the transduction channels of the outer hair cells [11 12]. The hearing loss caused by ototoxicity is progressive and irreversible and, with nephrotoxicity, is the main limiting factor of the CDDP dosage in current clinical therapeutic strategies.

Traditionally, in experimental animals, the overall alteration of the hearing threshold, due to cisplatin administration, has been studied by means of auditory brainstem responses (ABR) [4 5 7 13 14]. These measurements represent the integration (contribution) of individual responses from many neural fibres, therefore minute changes in cochlear micro-mechanics, caused by possibly transitory ototoxic effects, are not revealed. A detailed description of

eventual dysfunction in cochlear micro-mechanics caused by cisplatin ototoxicity can be obtained via recordings of the otoacoustic emissions (OAEs) [8 15]. These are considered responses of cochlear origin, generated when the auditory periphery is stimulated by a click or a pure tone stimulus and their close relationship with the non-linear micro-mechanics of the outer hair cells has been well established. In this context, use of OAEs can establish not only the presence of an ototoxic effect, but also evidence regarding the progress of ototoxicity as seen from the perspective of the OHCs.

Species differences between Humans and experimental animals exist in the susceptibility of the inner ear. In general, doses inducing an ototoxic effect of cisplatin in experimental animals exceed the doses used in the treatment of patients. It has been demonstrated, in several species, that there is a significant individual variability of hearing loss related to cisplatin treatment [15]. Even though several parameters, such as the pharmacokinetic pattern and pre-treatment hearing status, have been taken into consideration [7], no predictive factor for cisplatin-induced hearing loss has been identified.

The fact that a CDDP ototoxic insult results in the loss of outer hair cells due to apoptotic mechanisms has generated great clinical interest for substances: i. which might protect the inner ear from CDDP; and ii. which do not interfere with the activity of the anti-neoplastic agent. Animal studies [1 4 6 16] and clinical observations [1 17] have demonstrated that the family of thiosulphate compounds can protect from platinum ototoxicity including the drug carboplatin (a newer platinum compound). For the latter, Muldoon et al. [18] have shown that administration of sodium thiosulphate, following carboplatin treatment, significantly reduces ototoxicity in guinea pigs, times and doses being consistent with the anti-tumoural activity. In previous investigations [8 15], we evaluated the toxic effects, induced by CDDP, in the Sprague-Dawley rat model, by means of electrophysiological and morphological studies of the cochlea. From these studies, it was concluded that hearing loss related to apoptosis of the outer hair cells can be reliably predicted by otoacoustic emission measurements. In the present study, we evaluated the protective efficacy of a systemic administration of D-methionine using, as measuring technique, otoacoustic emissions (OAEs) verified by auditory brainstem responses (used as gold standard) [19].

Material and methods

A total of 12 male Sprague-Dawley albino rats have been used, mean weight 200 ± 20 g. Animals were divided into two groups: Group 1 (n = 8), and Group 2 (n = 4). Group 1 animals were treated with D-methionine (300 mg/kg), prior to the 30 min slow cisplatin infusion (16 mg/kg). Group 2 animals were considered as controls and received an equal volume of saline solution (instead of D-methionine) and cisplatin.

The experiment was performed as follows:

Group 1 animals:
- anaesthesia (ketamine/xylazine 1 ml/kg);
- administration of D-methionine ip (300 mg/kg);
- pre-treatment auditory function evaluation tests (ABR, OAEs);
- 30 min infusion ip of CDDP (16 mg/kg).

Group 2 (controls):
- anaesthesia (ketamine/xylazine 1 ml/kg);
- saline administration (volume equal to that of D-methionine administration in Group 1 animals);
- pre-treatment auditory function evaluation tests (ABR, OAEs);
- 30-min infusion ip of CDDP (16 mg/kg).

Post-treatment auditory function evaluation tests were performed, in both groups, 72 hours after CDDP administration.

The anaesthesia solution contained 1 ml of ketamine (100 mg/ml, Ketavet 100, Intervet, Aprilia, Italy), 1 ml of xylazine (20 mg/ml, Rompun, Bayer Leverrkuen, Germany), and 1 ml of saline. This solution was injected into the rat in 2 phases: in the first phase, a 1 ml/kg was administered by ip injection, and when the animal had reached, or shown signs of muscular relaxation another 0.5 ml/kg dose was administered under the skin (second phase).

CDDP was administered as 16 mg/kg body weight of the rat, at a concentration of 1.0 mg/ml. To simulate a clinical context, cisplatin was administered as a slow 30-min infusion using a Harvard apparatus micro-pump.

OAEs

Distortion product otoacoustic emission recordings

Distortion product otoacoustic emission (DPOAE) was recorded in a soundproof box by means of a Starkey 2000 device (Starkey Labs, Eden Praire MN, USA). The DPOAE analysis was set at a 4.0-16.0 kHz band (referring to f2), and 12 points per octave were sampled. The primary tone ratio was fixed at 1.21. The DPOAE responses were evoked by three non-symmetrical DPOAE protocols characterized by $L_1 > L_2$. Such protocols are generally considered the best choice to identify cochlear dysfunction [20 21]. The protocols used were defined as follows: P1 = low level (L1 = 40 and L2 = 30 dB SPL); P2 = middle level (L1 = 50 and L2 = 40 dB SPL); P3 = high level (L1 = 60 and L2 = 50 dB SPL).

During the DPOAE recordings, the body temperature was maintained at 37 ± 0.5 °C by a temperature con-

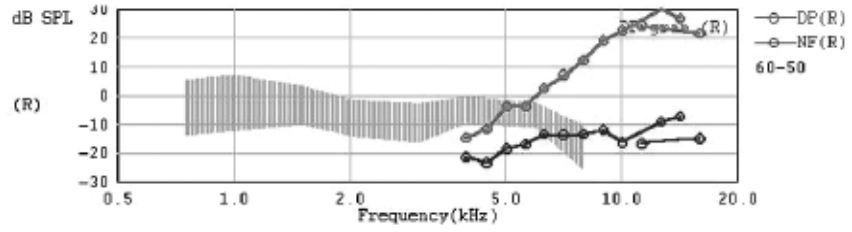

Fig. 1. Typical DPOAE response of Sprague-Dawley rat. Upper curve: DPOAE amplitude; lower curve: noise floor.

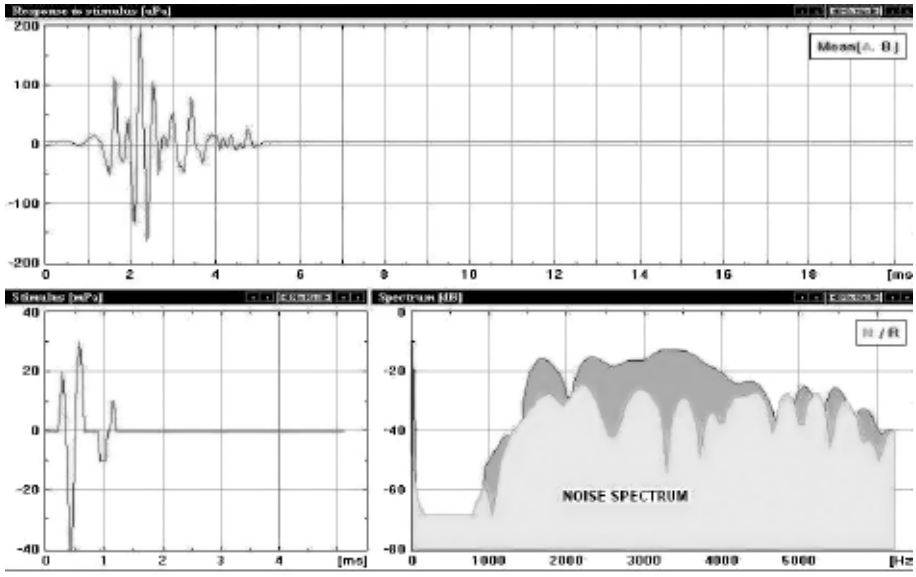

Fig. 2. Typical TEOAE response of Sprague-Dawley rat; response, stimulus and TEOAE spectrum are plotted (dark gray area of spectrum: response; light gray: noise).

trol device (Harvard Apparatus, Holliston MA, USA). A typical DPOAE response is shown in Figure 1.

TEOAE RECORDINGS

To optimize the transient evoked otoacoustic emission (TEOAE) recordings, the anaesthetized animal was placed under a stereotaxic device which held, without any movement, a neonatal ILO probe. This was introduced in the right external acoustic meatus through a small tube (diameter 3 mm) 35 mm in length.

Recordings of the TEOAE were made in a soundproof box by ILO-92 (Otodynamics Ltd, Herts**,** UK), at the beginning of the experiments (time = 0) and again 72 hours after CDDP administration. The TEOAEs were evoked by a 80 µs click of an intensity 63 ± 2 dB p.e. SPL, according to the standard nonlinear ILO protocol [18]. To eliminate residues related to a stimulus artefact, data have been analysed with a temporal window ranging from 1.5 to 4.5 ms. The TEOAE responses were evaluated in the frequency domain (FFT), by estimating signal to noise ratios (SNR) at 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 kHz.

During the TEOAE recordings, the animal's body temperature was maintained at 37 ± 0.5 °C by a temperature control device (Harvard, USA). A typical TEOAE response is shown in Figure 2.

AUDITORY BRAINSTEM RESPONSES

The procedure for recording Auditory Brainstem Responses (ABRs) in the Sprague-Dawley rat has been described elsewhere [8 15]. Briefly, the ABRs were recorded by means of three platinum-iridium needle

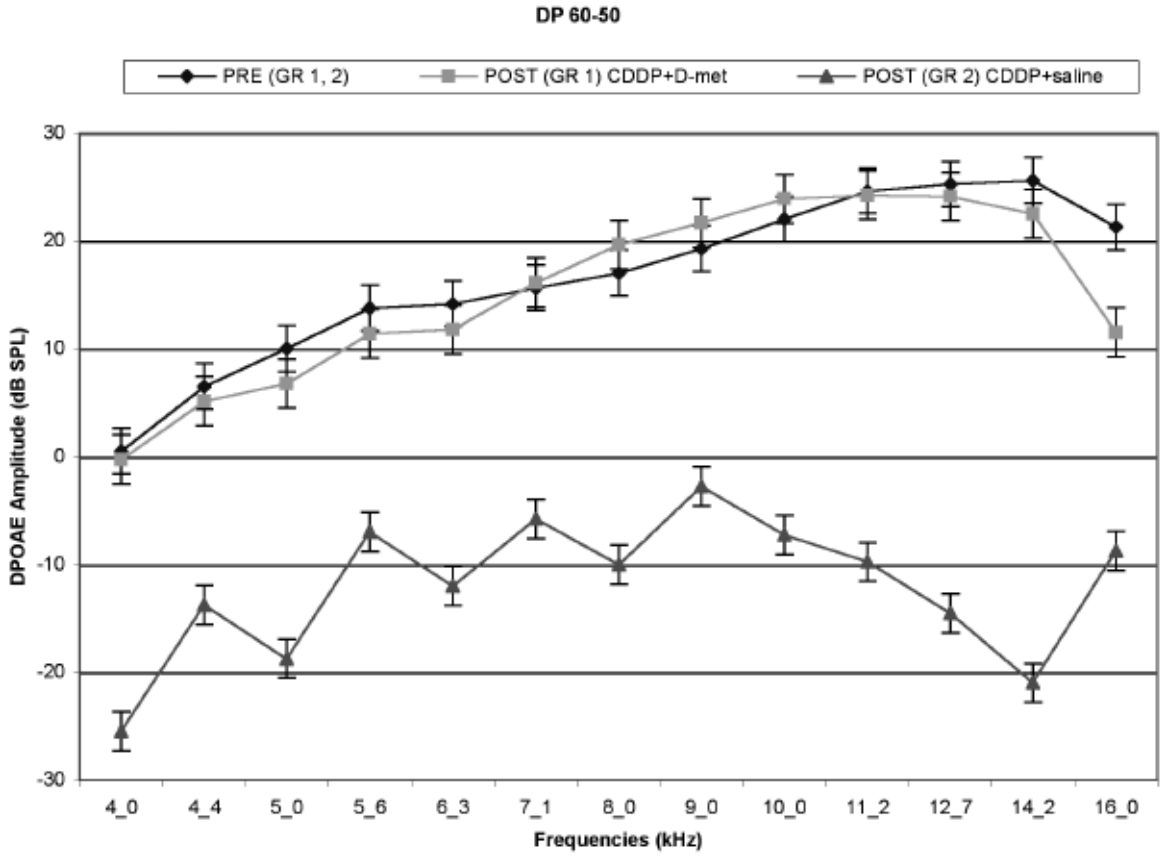

Fig. 3. High stimulus protocol (60-50 dB SPL) average DP-GRAMs. Top curve (rhombus): mean pre-treatment values for all tested animals (n = 12). Top curve (squares): post-treatment values of Group 1 (n = 8) data. Lowest curve (triangles): post-treatment data from Group 2 (controls; n = 4). Significant amplitude differences were observed at all tested frequencies.

Table I. Comparison (t test) of DPOAE (60-50)responses between recorded data of animals. Three pairs were tested: Group 1 (protected) post-treatment *vs.* pre-treatment values, Group 2 (controls, not protected) post-treatment *vs.* pre-treatment values and Group 1 and Group 2 values after cisplatin treatment. Significant differences were found ($p < 0.05$).

Paired Samples Test

		Paired Differences					t	df	p value (2-tailed)
		Mean	SD	SE Mean	95% CI of Difference Lower	Upper			
Pair 1	PRE-GR2_POST	1.3173	3.2180	0.8925	-0.6273	3.2619	1.476	12	0.166
Pair 2	PRE-GR1_POST	28.6346	7.7863	2.1595	23.9294	33.3398	13.260	12	0.000
Pair 3	GR1_POST-GR2_PRE-	27.3173	7.7136	2.1394	22.6561	31.9786	12.769	12	0.000

electrodes, placed subdermally over the vertex (positive), the mastoid (negative) and the dorsum area (reference/ground) of the animal. The recordings were made in a sound-treated cabin, the walls and ceiling of which were covered by phono-absorbent material. Calibration of the sound field was achieved

Table II. Comparison (t test) of TEOAE values between recordings of Group 1 animals. Values pre- and post-treatment were assessed for the following parameters: reproducibility (REPRO), response (RESP), signal to noise ratio at 1, 2, 3, 4, 5, 6 and 7 kHz (SN1, SN2, SN3, SN4, SN5, SN6 and SN7, respectively).

		Independent Samples Test								
		Levene's test for Equality of Variances		t-test for Equality of Means						
									95% CI of Difference	
		F	Sig.	t	df	p. value (2-tailed)	Mean Difference	SE Difference	Lower	Upper
REPRO	Equal variances assumed	2.490	0.141	1.066	12	0.307	6.0000	5.6291	-6.2648	18.2648
	Equal variances not assumed			1.066	9.973	0.312	6.0000	5.6291	-6.5471	18.5471
RESP	Equal variances assumed	0.553	0.471	1.403	12	0.186	2.9429	2.0981	-1.6285	7.5142
	Equal variances not assumed			1.403	11.579	0.187	2.9429	2.0981	-1.6470	7.5327
SN1	Equal variances assumed	0.000	0.997	-0.326	7	0.754	-0.8500	2.6098	-7.0212	5.3212
	Equal variances not assumed			-0.323	6.350	0.757	-0.8500	2.6311	-7.2029	5.5029
SN2	Equal variances assumed	0.134	0.722	0.820	10	0.432	2.4857	3.0326	-4.2712	9.2427
	Equal variances not assumed			0.785	7.372	0.457	2.4857	3.1657	-4.9241	9.8955
SN3	Equal variances assumed	0.942	0.351	0.620	12	0.547	1.7143	2.7664	-4.3132	7.7418
	Equal variances not assumed			0.620	10.300	0.549	1.7143	2.7664	-4.4254	7.8540
SN4	Equal variances assumed	0.307	0.590	0.720	12	0.486	1.5714	2.1837	-3.1865	6.3294
	Equal variances not assumed			0.720	11.653	0.486	1.5714	2.1837	-3.2023	6.3452
SN5	Equal variances assumed	0.001	0.979	-0.539	12	0.600	-2.0000	3.7088	-10.0808	6.0808
	Equal variances not assumed			-0.539	11.998	0.600	-2.0000	3.7088	-10.0809	6.0809
SN6	Equal variances assumed	0.170	0.691	0.319	8	0.758	0.5160	1.6197	-3.2191	4.2511
	Equal variances not assumed			0.319	8.000	0.758	0.5160	1.6197	-3.2191	4.2511
SN7	Equal variances assumed	3.932	0.095	0.756	6	0.478	49.1920	65.0677	-110.0229	208.4069
	Equal variances not assumed			1.008	4.001	0.370	49.1920	48.8025	-86.2947	184.6787

using a Bruel & Kjaer (Naerum, Denmark) microphone (type 2209), placed 4 cm above the animal's head and facing the loudspeaker.

ABRs were amplified 20000-fold and filtered from 20 to 5000 Hz. Each recording was the average of 500-1000 individual responses. The ABRs were generated in response to 100 µs alternated clicks and 8, 10, 16, 20, kHz tone pips (1 ms rise-fall time, 10 ms plateau), in the range 100-30 dB SPL. The sound transducer, a Motorola (Schamburg IL, USA) tweeter (flat response ± 1 dB from 4.0 to 35 kHz), was placed 4 cm away from the rat's ear. At the minimum

Table III. ABR threshold shifts in dB of ABR responses from the two groups.

Frequency (kHz)	Group 1 (dB SPL)	Group 2 (dB SPL)
8	10 ± 5	30 ± 5
10	10 ± 5	30 ± 5
16	10 ± 5	25 ± 5
20	15 ± 10	45 ± 10

Results

Analysis of the DPOAE recordings of the three protocols (L3: 60-50, L2: 50-40, L1: 40-30 dB SPL) suggested that all untreated animals (Group 2) presented a significant reduction of the DPOAE amplitude. The latter affected the entire spectrum of the frequencies considered (4-16 kHz). The most significant differences were observed in the recordings from the L3 protocol (60-50 dB SPL) DPOAE. These data are shown in Figure 3 and analysed in Table I.

The protected animals from Group 1, presented DPOAE amplitudes which showed no significant differences between the pre- and post-treatment recordings. It should be pointed out, however, that the DPOAE amplitudes from the post-cisplatin DPOAE recordings were characterized by lower values. The TEOAE amplitude and the TEOAE spectra (measured at 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 kHz) were significantly impaired in Group 2 (data not shown), while no significant differences were observed in Group 1, between the pre- and post-recording data (Tab. II).

The ABR data from the unprotected group presented threshold shifts reaching 45 ± 10 dB SPL at 20 kHz and 25 ± 10 dB SPL at 16 kHz and 30 ± 10 dB SPL both at 10 and 8 kHz (a Table with the threshold

Table IV. Mean threshold ABR values from Group 1 (protected animals).

Frequency (kHz)	Pre-treatment (dB SPL)	Post-treatment (dB SPL)	p value
8	35 ± 5	45 ± 5	n.s.
10	35 ± 5	45 ± 5	n.s.
16	40 ± 5	50 ± 5	n.s.
20	40 ± 10	55 ± 10	n.s.

* = $p < 0.05$; n.s. = not significant.

Table V. Mean threshold ABR values from Group 2 (unprotected animals).

Frequency (kHz)	Pre-treatment (dB SPL)	Post-treatment (dB SPL)	p value
8	35 ± 5	65 ± 5	*
10	35 ± 5	65 ± 5	*
16	40 ± 5	65 ± 5	*
	20	40 ± 10	85 ± 10 *

* = $p < 0.05$

threshold level, two recordings were acquired. No responses were present below a stimulus level of 30 dB SPL, which was considered the threshold level for our experimental set-up. During all measurements, the animal's body temperature was maintained at 37 ± 0.5 °C by a rectal probe connected to a Harvard Apparatus homeothermic blanket. Ear plugs were used to occlude the contra-lateral ear in order to avoid a binaural stimulation at high stimulus intensities.

Statistical analysis

Statistical analyses were performed using Student's t test ($p < 0.05$).

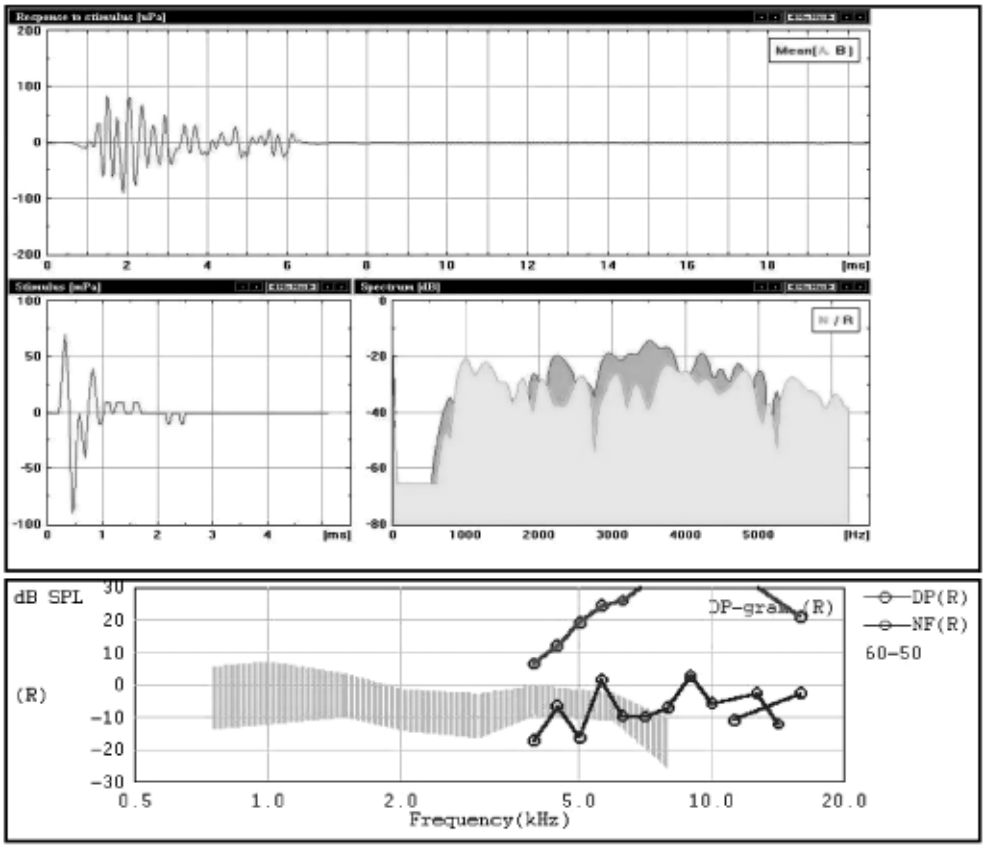

Fig. 4. Group 1. Pre-recordings before cisplatin administration (error bars have been omitted). Top panel: mean TEOAE response. Central panels: mean stimulus response and mean TEOAE spectra. Lower panel: mean DP-GRAM.

shifts is also presented in the Appendix). The ABR responses presented small threshold shifts of 15 ± 10 dB at 20 kHz and less than 10 ± 5 dB SPL at 8.0, 10 and 16 kHz. The differences at 20 kHz reached borderline significance (Tables III-V).

The otoprotective effects of D-methionine are summarized in Figures 4-6. Figure 4 shows the mean pre-administration TEOAE and DPOAE data from Group 1. Figure 5 shows the post-administration TEOAE and DPOAE data. Some structural differences were observed in the TEOAE response but the spectral estimate differences remained non-significant. The DPOAE post-administration responses showed a steeper slope at high frequencies (i.e., > 8.0 kHz) but the post-/pre-differences remained non-significant. Figure 6 shows the mean post-administration data from Group 2. A comparison with data in Figures 4 and 5 indicates structural differences in the TEOAE responses and signal-to-noise ratio differences in the DPOAE responses (in this case, the responses are very close to the noise floor).

Discussion

Clinical use of chemoprotectors has always been limited due to the negative interactions with the chemotherapeutic drugs, thus reducing their effectiveness on the neoplastic processes. In this context, it is very important to establish whether D-methionine interferes with the anti-tumoural action of CDDP. Some studies have shown that *in vivo* the anti-tumoural action does not decrease following pre-administration of D-methionine [8 16 17 19] and similar results have been reported in ovarian cancer *in vivo* [14]. Controversial results have been obtained in an *in vitro* study on the simultaneous administration of CDDP and D-methionine, demonstrating an effective reduction of the anticancer action of cisplatin [22].

As revealed by Reser et al. [19], sulphurs containing antioxidants have the potential to compensate the side-effects of CDDP, and it has been observed that both isomers of sulphur methionine block the CDDP toxic effects in the ear and in the kidney. The D-methio-

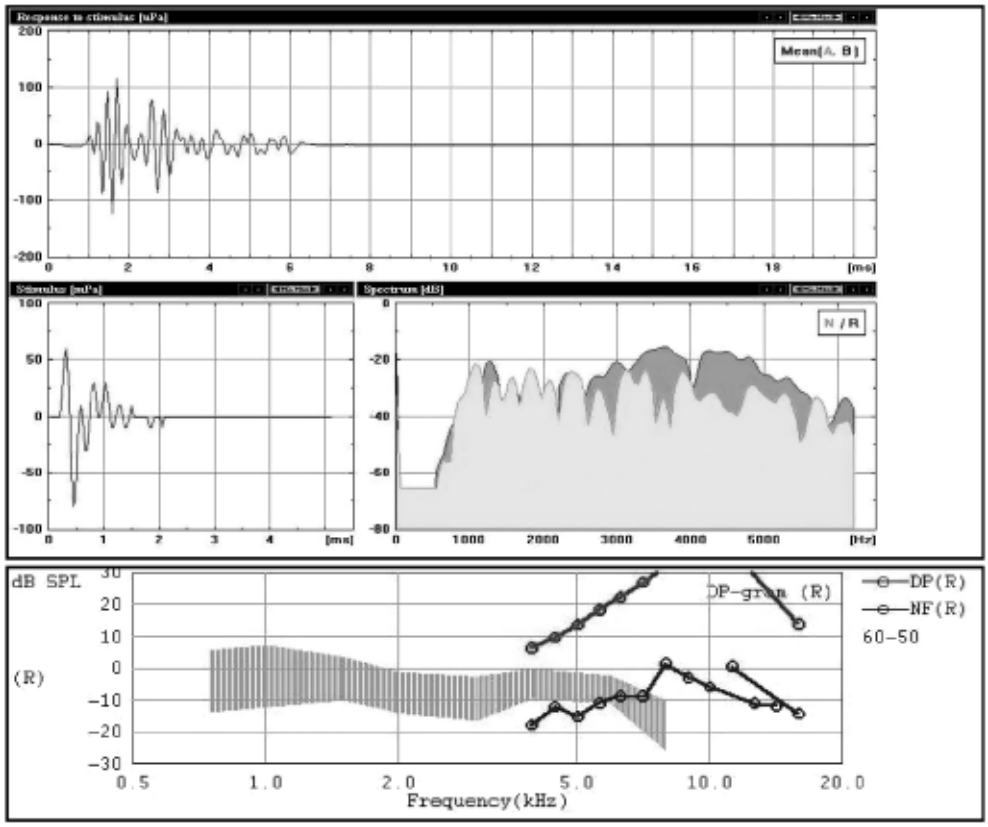

Fig. 5. Group 1. Post-recordings after cisplatin administration (structure of figure, same as Fig. 4). Several structural differences are evident in TEOAE response and spectrum but pre/post differences were not statistically significant. DPOAE maintained same waveform as pre-recordings (Fig. 4).

nine-treated animals [19], following CDDP administration, did not present auditory damage, according to the post-treatment ABR recordings, and the data from the electron microscopy scan of the cochlea. Campbell et al. [4 13] evaluated the use of D-methionine in a Wistar rat model. The animals received 16 mg/kg CDDP, and various dosages (75, 150 and 300 mg/kg) of D-methionine, 30 minutes before and 3 days after the treatment. The ABR data suggested that animals which received 300 mg/kg D-methionine presented good to full otoprotection.

The fact that data presented in the literature favour both procedures, suggests that the administration of a chemoprotector should not be given with the same modality as cisplatin administration (i.e., i.v.). Several more recent studies, using laboratory animals, have supported the hypothesis that to reliably treat the inner ear, a chemoprotector should be released locally, administered through the round window [23 24]. Although such an approach has beneficial effects on the efficacy of oto-protection and the anti-tumoural efficacy of cisplatin, it, nevertheless, raises the question of the type of technology needed to minimize the traumatic administration of a chemoprotector in the standard audiological setting.

This study tested the hypothesis that the administration of antioxidant agents could prevent CDDP-induced hearing loss. The results obtained suggest that the systemic administration of D-methionine has a potential oto-protective role. Data from the OAE recordings suggest good recovery of the post-treatment responses. Albeit, the post-cisplatin OAE data did not demonstrate 100% recovery. This implies that, in the tested conditions (very high cisplatin dosage), D-methionine can partially protect the outer hair cells. The data from the ABR recordings at 20 kHz also support this hypothesis. It could be argued that the cisplatin dosage tested exceeds many times the dosages used in clinical practice. In this context, it is feasible that D-methionine can protect, completely and more efficiently, the inner ear at lower dosages of cisplatin (i.e., 6-12 mg/kg) but further data are necessary to confirm this hypothesis.

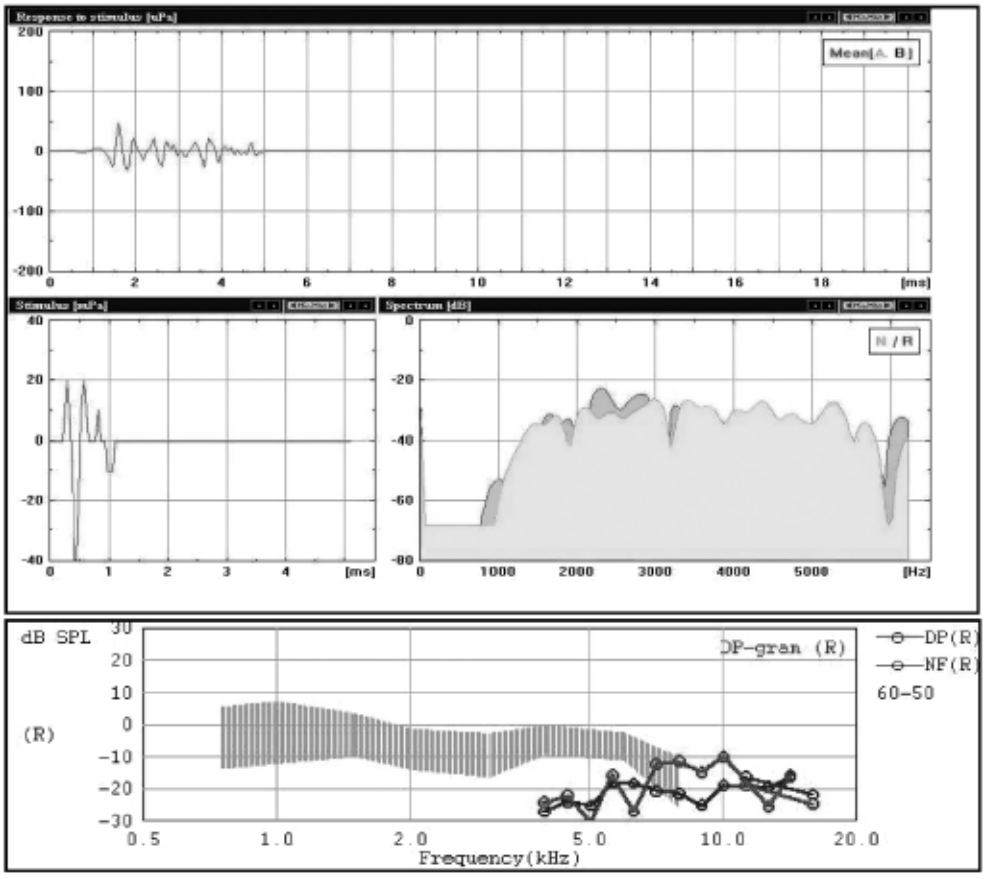

Fig. 6. Group 2 (control). Post-recordings after cisplatin administration (structure of figure, same as Fig. 4). TEOAE response shows fewer components and a significantly reduced amplitude (main peak < 50 uPa). TEOAE spectrum shows that most frequencies are within the noise-floor. DP-GRAM data verify TEOAE profile (frequencies < 8 kHz are within noise floor) and only from 8.0 to 10.0 kHz are responses above noise. These responses, however, are very small (amplitudes < 10 dB SPL).

Note: The figures shown in this article were prepared using in-house software (ILO-viewer) for the processing of TEOAEs. The software was developed as a collaborative project between the Polytechnic of Warsaw, Poland (Dr. Grzanka) and Ferrara University (Dr. Stavros Hatzopoulos). This software can be downloaded, free of charge, from the Otoacoustic Emissions Portal (http://www.otoemissions.org).

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