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Ototoxic effects of cisplatin in a Sprague–Dawley rat animal model as revealed by ABR and transiently evoked otoacoustic emission measurements

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Abstract

The ototoxic effects of cisplatin in a Sprague–Dawley rat model were evaluated by recordings of auditory brainstem responses (ABR) and transiently evoked otoacoustic emissions (TEOAEs). The ABR responses were evoked from alternating clicks and 8, 10, 12, 16, 20 and 30 kHz tone pips in a range from 40 to 100 dB SPL range. The TEOAEs were recorded with a non-linear protocol, and were evoked by a 63.5 dB SPL click stimulus. Twenty five male Sprague–Dawley rats were used in the study, 20 animals were treated with cisplatin (16 mg/kg, body weight) and five animals served as controls. The data showed that 72 h after the cisplatin administration, the TEOAE and ABR variables were significantly altered. The relationship between the ABR and TEOAE variables was shown to be non-linear. The most significant relationships were observed between the TEOAE correlation and the ABR threshold values at 10, 12, and 16 kHz.

Key words: Ototoxicity; Cisplatin; Otoacoustic emissions; Transiently evoked otoacoustic emission; Auditory brainstem response

1. Introduction

Cisplatin is a anti-neoplastic agent which is widely used in combination chemotherapy and it is known to generate a long series of side effects such as nephrotoxicity (Gandara et al., 1991; Kersten et al., 1998; Husain et al., 1998; Ueda et al., 1998) and ototoxicity (Fausti et al., 1984, 1992; Blakley et al., 1994; Saito and Aran, 1995; Ravi et al., 1995; Riggs et al., 1996; Campbell et al., 1996; Kohn et al., 1988). In patients receiving a high-dose treatment, cisplatin typically induces first a high-frequency hearing loss, which can gradually extend to lower frequencies during subsequent treatments (Kopelman et al., 1988; Laurell and Jungnelius, 1990; Laurell and Bagger-Sjöbäck, 1991). Cisplatin has multiple toxic effects on the guinea pig and rat cochlea, with a specific toxic effect on the outer hair cells (OHCs) (Stengs et al., 1998a; Taudy et al., 1992) and the stria vascularis (Blakley et al., 1994; Riggs et al., 1996; Campbell et al., 1996; Kohn et al., 1988; Tange and Vuzevski, 1982; Tange et al., 1982).

Traditionally, in experimental animals the overall alteration of the hearing threshold due to a cisplatin administration has been studied by the use of auditory brainstem responses (ABR) (Fausti et al., 1992; Ravi et al., 1995; Riggs et al., 1996; Campbell et al., 1996; Stengs et al., 1998a; Taudy et al., 1992). These measurements represent the integration (contribution) of individual responses from many neural fibers, therefore

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minute changes of the cochlear micromechanics, caused by possibly transitory ototoxic effects, can not be revealed. A possibly detailed description of dysfunction in cochlear micromechanics (Stengs et al., 1998b; Beck et al., 1992) caused by cisplatin ototoxicity can be obtained via recordings of the otoacoustic emissions (OAEs). 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 micromechanics of the OHCs has been well established (McFadden and Plattsmier, 1984; Brownell et al., 1985; Brownell, 1990). Within this context, it can be said that the use of OAEs can provide not only a verification of the presence of an ototoxic effect, but evidence regarding the progress of the ototoxicity as seen from the perspective of the OHCs.

Species differences between man and experimental animals exist in the susceptibility of the inner ear. In general, doses inducing an ototoxic effect of cisplatin in experimental animals exceed widely 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 in connection to cisplatin treatment (Saito and Aran, 1994; Riggs et al., 1996; Hatzopoulos et al., 1999; Sochalingam et al., 2000). Even though several parameters such as the pharmacokinetic pattern (Ekborn et al., 2000) and pre-treatment hearing status (Kopelman et al., 1988) are taken into consideration, no predictive factor for cisplatin-induced hearing loss has been identified.

Recordings of transiently evoked otoacoustic emissions (TEOAEs) from the rat have been reported recently (Khvoles et al., 1996, 1998; Hatzopoulos et al., 1999; Sochalingam et al., 2000), using commercial clinical equipment from Otodynamics and Madsen. The recorded signals are characterized by small latencies in the order of 1.0–2.5 ms and average amplitudes (200–300 μPa). The rat TEOAE recordings have been described as stable and repetitive, as the recordings from human subjects and constitute an ideal non-invasive method to study the perturbation of the cochlear micromechanics.

A number of previous studies have used a TEOAE–ABR approach to study the ototoxic effects of cisplatin, but the data reported refer either to low cisplatin doses (Hatzopoulos et al., 1999) or to responses evoked by click stimuli (Sochalingam et al., 2000). Important information on the relationship between TEOAEs and ABR responses, evoked by mid- to high-frequency tone pips, have been left undefined. Considering that TEOAE-based cochlear testing is the most used OAE protocol in human studies and clinical hearing evaluations, it is important to define the type of information that a TEOAE protocol provides. In addition there are clinical motives to search for an alternative test to identify ototoxic effects. First, it would be useful to have a reliable and objective method for an early detection of ototoxicity. Second, in small children and severely affected patients routine psychoacoustic tests cannot be performed.

The objectives of this project were the following: (1) the validation of the hypothesis that the TEOAEs can be used as good indicators of cochlear function/dysfunction after administration of cisplatin; (2) the identification of the TEOAE parameter(s) which describe better the induced ototoxic effects; and (3) the study of the relationship between the TEOAE variables and the ABR threshold values 8–20 kHz, as a means to obtain additional information on the frequency specificity of the TEOAE protocol.

Several assumptions were made regarding the objectives of the study. First, we have used a Sprague–Dawley rat animal model treated with 16 mg/kg of cisplatin. The potent ototoxic effect of 16 mg/kg body weight (b.w.) cisplatin in the rat has been shown to deteriorate the electrophysiologically hearing thresholds (Meech et al., 1998; Ravi et al., 1995) as well as to induce a reduction of the endocochlear potential (Saito and Aran, 1995). Second, we have assumed that the TEOAEs could be used to verify functional cochlear changes located at higher frequencies, than the ones present in the spectrum of the TEOAE signal (Withnell et al., 1998; Withnell and Yates, 1998). We expected that early ototoxic effects (evaluated 72 h post-treatment) would be manifested as a significant decrease of the spectral content of the TEOAE recordings. The ABR were used as validators (golden standard) of the induced ototoxic effects.

2. Materials and methods

2.1. Chemicals

Cisplatin used in the animal treatments was Platinex from Bristol Myers (0.5 mg/ml in normal saline) which is the product used clinically in Italy. Cisplatin was administered to anesthetized animals, according to the protocol guidelines presented below.

For the animal anesthesia an equal-volume combination of ketamine (Ketavet, Farmaceutici Gellini, Italy), xylazine (Rombun, Bayer, Italy) and saline were used in dosages of 1 ml/kg of b.w. Each ml of anesthesia contained 33.3 mg of ketamine and 6.7 mg of xylazine/kg b.w. The anesthetic was administered in two consecutive phases. In phase one, the animal received a 1 ml/kg b.w. intra-peritoneal dose and upon the first signs of muscular relaxation (phase two) a second halved-volume dose was administered subcutaneously.

2.2. Animals

Twenty five male Sprague–Dawley rats obtained from Charles River Italy (mean weight 210 ± 30 g) were divided randomly into two groups. Twenty animals were treated with 16.0 mg/kg of cisplatin and five animals received an equivalent volume of saline and they were used as controls. The cisplatin was administered by an intra-peritoneal slow infusion (post-anesthesia) of about 30 min using a micro-pump from Harvard Apparatus. To avoid extensive dehydration (a cisplatin side-effect) the animals were extra-hydrated daily with saline solution which was administered orally. The animals were treated according to the Italian guidelines DL 116/92 with reference to EEC directive number 86-609.

2.3. Electrophysiological studies

The ABR and TEOAE responses were recorded, during anesthesia, before and 72 h after the cisplatin administration. In the pre-treatment phase the animals were (i) anesthetized, (ii) the TEOAE and ABR responses were acquired and (iii) the cisplatin treatment was conducted.

2.3.1. TEOAEs

The TEOAE signals (in the ILO terminology a TEOAE signal is called TEOAE 'response', a term which will be used in the rest of this article) were recorded in a sound-proof cabin by the ILO-292 apparatus. An in situ calibration was conducted on the ILO transducer prior to any recording. A click was presented to the transducer and the resulting SPL levels were memorized. Based on these readings the ILO-292 output levels were equalized for each tested animal.

The TEOAEs were evoked by a 80 μs click stimulus of 63 ± 2 dB peak equivalent SPL, following a non-linear protocol (a stimulus train composed of four clicks: three positive and one negative with an amplitude 9.5 dB higher than the positive clicks). Each TEOAE response was the average of 1000 stimuli, which were presented at a rate of 50 rep/s. The length of each TEOAE recording was 20.4 s. The data were initially acquired with a 1.0–19.5 ms window (note: the default ILO values are 2.5–19.5 ms). To avoid a probable contamination of the TEOAE 'response' by the trail of the acoustic click stimulus, the initial 1.5 ms of each recording were removed by a cosine window function (rise time 0.64 ms). Since the Sprague–Dawley rats demonstrate short-latency responses which do not exceed 5 ms, the TEOAE segments after 5 ms were also suppressed using a cosine window function with the characteristics of the one mentioned above. For the analyses of the TEOAE data we have considered only the recording segment from 1.5 to 5 ms. The TEOAE responses were transformed by a fast Fourier algorithm and signal to noise ratio (S/N) estimates were calculated at 1.5, 2.0, 3.0, 4.0, and 5.0 kHz.

In order to record the TEOAE response, the anesthetized animal was placed under a stereotaxic device. A neonatal ILO probe was connected to the external meatus of the animal by a thin tube of 35 mm length and 3 mm diameter. A tight seal was possible when the tube was inserted on the average 5 mm into the external auditory meatus. The tube connecting the probe and the acoustic meatus of the animal, is considered an acoustic filter attenuating high frequencies. In our experimental context this is not a problem, because the ILO apparatus already attenuates the TEOAE high frequencies above 5 kHz by 80 dB/decade. The reader should note though, that according to the data from a previous study (Withnell et al., 1998), the high TEOAE frequencies are expressed as low-frequency intermodulation distortion products, which lie into the pass-band of the ILO hardware.

For the TEOAE data visualization a special in-house software package was developed (see note 1). For the data analysis we have used ILO software version 5.6.

2.3.2. ABR

The ABR responses were recorded by three platinum–iridium needle 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 whose walls and ceiling were covered by phono-absorbent material. The calibration of the sound field was done using a Bruel and Kjaer microphone (type 2209), placed 4 cm above the animal's head and facing the loudspeaker.

The ABRs were amplified 20 000 times 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, 12,16, 20, and 30 kHz tone pips (1 ms rise–fall time, 10 ms plateau), in the range 100–30 dB SPL. The sound transducer, a Motorola tweeter (flat response ± 1 dB from 4.0 to 35 kHz), was placed 4 cm away the rat's ear. Threshold was based on the visibility and reproducibility of wave III, according to Bourre et al., 1999. At the minimum threshold level two recordings were acquired. No responses were present below a stimulus level of 40 dB SPL, which was considered the threshold level for our experimental set-up. During all measurements the body temperature of the animal was maintained at 38 ± 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.

2.4. Statistical analyses

For each of the TEOAE variables (response, correlation, S/N estimates in 1.5, 2.0, 3.0, 4.0 and 5.0 kHz bands) post- minus pre-treatment differences were obtained. The means of these differences were compared using a confidence level of 0.95. The most sensitive TEOAE variable, which best characterizes the post–pre TEOAE alterations, was identified as the one having the smallest *t*-test probability value or, equivalently, since the sample sizes were all equal, the greatest *t*-statistic magnitude.

In order to identify the TEOAE variable(s) which relate better to the ABR threshold values, at mid- to higher stimulus frequencies, we conducted a regressions between the TEOAE variables (used as the predictors) and the ABR values (used as the responses). The reader should notice that the ABR and the TEOAE methodologies measure different aspects of the ototoxic insult. Although in traditional regression analysis one compares methods using similar stimulation schemes, in this experimental set-up this was not feasible due to the bandwidth limitations of the ILO-292. Nevertheless, the objective of the regression analysis was to relate the ABR threshold shifts with a set of TEOAE variables, based on the hypothesis that high-frequency TEOAE information (corresponding to the frequencies at which ABR responses were acquired) is translated into lower octaves, recordable by the ILO-292.

In the regression analyses we have considered two possible scenarios: (a) in the first scenario we have considered differenced ABR and TEOAE variables, using the post- minus pre-treatment values of these variables; (b) in the second scenario we have considered the post-treatment ABR and TEOAE values. To increase the predictability of the models for each scenario we have: (i) selected multiple regression models using a stepwise regression procedure; and (ii) we have estimated regression models of higher order (from linear to sixth order). The details and the rules employed of the model fitting are presented in the Appendix.

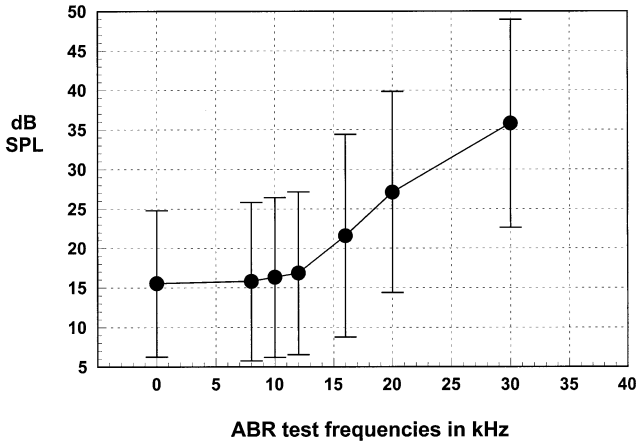

Fig. 1. ABR threshold shifts at the tested frequencies. The *y*-axis depicts shifts in dB and the *x*-axis shows the tested frequencies. In the graph the point on the *x*-axis indicated as '0' refers to the click threshold levels.

In order to describe more accurately the effects of cisplatin on the structure of the TEOAE responses, Pearson correlation coefficients and bootstrap BCa (bias-corrected and accelerated) confidence intervals were computed from the pre- and post-treatment TEOAE data. Detailed information on the novel bootstrap procedure can be found in the Appendix.

All analyses were implemented with a mainframe SAS package.

Table 1
Descriptive statistics of the TEOAE variables pre- and post-treatment values

	Number of animals	Mean	S.D.	Min.	Max.
Pre-treatment variables					
TEOAE response (dB SPL)	20	−1.39	3.26	−6.4	5.0
TEOAE correlation %	20	90.55	3.92	80.0	96.0
S/N 1.5 kHz (dB)	20	7.5	7.83	−1.0	28
S/N 2.0 kHz (dB)	20	10.2	4.56	0.0	17.0
S/N 3.0 kHz (dB)	20	11.55	4.11	3.0	17.0
S/N 4.0 kHz (dB)	20	8.45	5.20	−4.0	17.0
S/N 5.0 kHz (dB)	20	2.85	3.85	−5.0	9.0
Post-treatment variables					
TEOAE response (dB SPL)	20	−10.37	3.81	−15.0	−2.3
TEOAE correlation %	20	63.85	19.92	26.0	91.0
S/N 1.5 kHz (dB)	20	3.65	10.29	−15.0	25.0
S/N 2.0 kHz (dB)	20	3.5	6.27	−10.0	15.0
S/N 3.0 kHz (dB)	20	3.65	7.31	−15.0	16.0
S/N 4.0 kHz (dB)	20	−4.65	9.47	−15.0	10.0
S/N 5.0 kHz (dB)	20	−9–9	7.27	−15.0	3.0

The numbers 15, 20, 30, 40 and 50 refer to the S/N ratios at 1.5, 2.0, 3.0, 4.0 and 5.0 kHz.

3. Results

3.1. Description of the post-treatment ABR and TEOAE data

All animals treated with cisplatin (20 cases) presented a 72 h survival rate. After the acquisition of data the animals were sacrificed with an anesthesia overdose, according to the Italian guidelines of animal experimentation (DL 116/92). Only the right ear ABR and TEOAE responses were considered according to previous data (Hatzopoulos et al., 1999).

The ABR recordings from the cisplatin-treated animals presented significant mean threshold shifts of 35.8 dB at 20–30 kHz and threshold shifts of 16 dB at 8–16 kHz. The lowest mean threshold shift of 15.2 dB was observed for the click ABR responses. The mean electrophysiological post-treatment threshold shifts, per tested frequency, are shown in Fig. 1.

The post-treatment TEOAE responses presented a

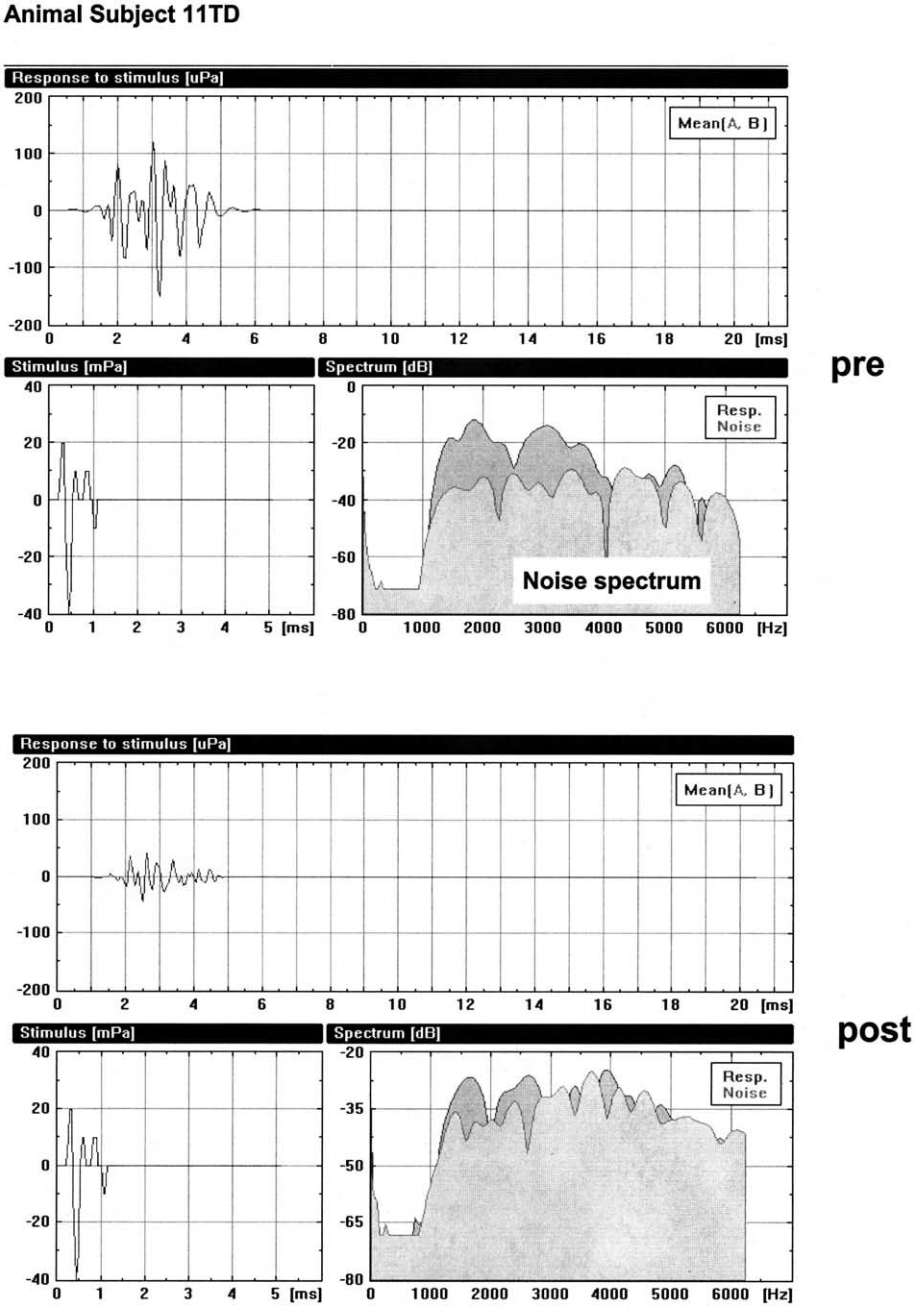

Fig. 2. TEOAE responses from the animal 11TD, (top) before and (bottom) after the cisplatin administration. The three panels (top and bottom) show the mean TEOAE response expressed in μPa (the horizontal axis depicts time in ms), the calibration stimulus and the cross TEOAE-spectrum in relative units. The spectral bandwidth has been limited to 6 kHz by the ILO software.

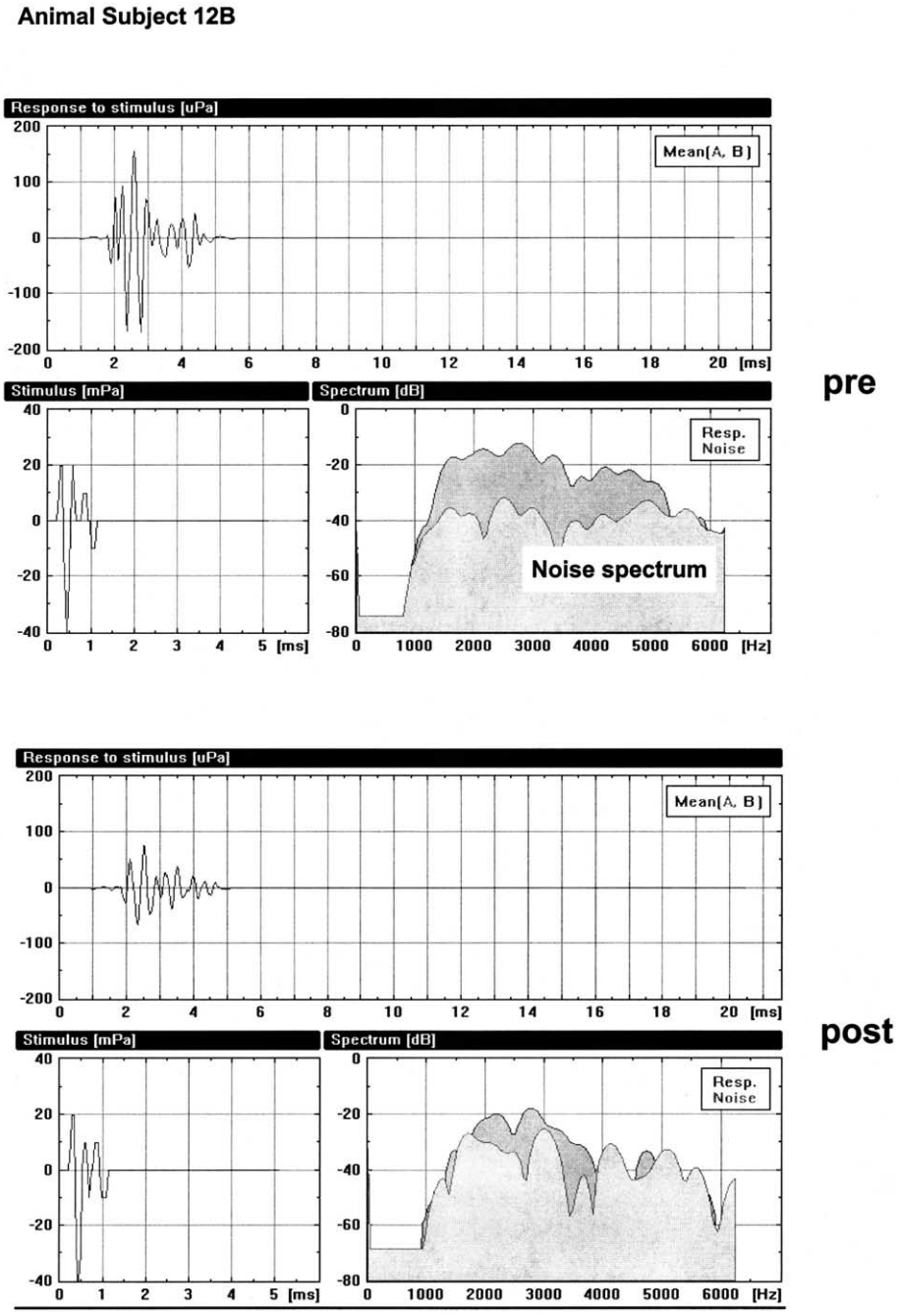

Fig. 3. TEOAE responses from animal 12B (top) before and (bottom) after the cisplatin administration. The format of the picture follows the one from Fig. 2.

suppression of the TEOAE amplitude and a shortening of the bandwidth of the response's spectrum (caused probably by the decrease of the TEOAE signal). These effects were subject-dependent and various degrees of TEOAE amplitude depression were observed. The larger value-decrease was observed for the S/N ratio at 5.0 kHz. Descriptive statistics of the pre- and post-TEOAE data sets are shown in Table 1. The majority of the variables presented skewed distributions. Fig. 2 presents data from an animal whose post-treatment TEOAE responses were almost missing. Fig. 3 shows data from another treated subject whose post-treatment TEOAE responses are suppressed but still visible.

3.2. *Reduction of the mean values of the TEOAE variables*

The 95% confidence intervals for the differences between the post- minus the pre-treatment TEOAE responses are shown in Table 2. Student's *t*-statistic

Table 2
The data in the table show the t-statistics on the differences obtained from the post minus the pre-treatment TEOAE datasets

Variable name	Interval values	*P*-value	Interpretation
TEOAE response (dB)	−10.861, −7.099	<0.0001	***
Correlation %	−36.051, −17.394	<0.0001	***
S/N ratio 1.5 kHz (dB)	−9.185, 1.485	0.1474	NS
S/N ratio 2.0 kHz (dB)	−10.017, −3.383	0.0005	***
S/N ratio 3.0 kHz (dB)	−11.579, −4.221	0.0002	***
S/N ratio 4.0 kHz (dB)	−18.224, −7.976	<0.0001	***
S/N ratio 5.0 kHz (dB)	−16.301, −9.199	<0.0001	***

*** = Significant effects, NS = non-significant effects. The columns show the TEOAE variables, the 95% interval values, the *t*-statistics, the corresponding probability values and the statistical interpretation.

indicates that the differences have means significantly different than zero, with the exception of the S/N ratio at 1.5 kHz.

From the variables presenting significant differences, the highest *t*-values (lowest *P*-values) were observed for the TEOAE response, S/N ratio at 5.0 kHz and TEOAE correlation. In this context, the TEOAE variable which best characterized the cisplatin-induced cochlear alterations was the TEOAE response. This variable was used in the regression analysis with the ABR threshold data.

3.3. The relationship between ABR and TEOAE variables

The results from the multiple regressions are shown in Table 3 and Tables A2 and A3 (for reasons of complexity, the data are presented in the Appendix, along with the explanations of the statistical parameters involved). The hypothesis to be evaluated was whether the TEOAE response was correlated with the ABR threshold levels at 8, 10, 12 and 20 kHz.

3.3.1. Post- minus pre-treatment data

For the post- minus pre-treatment data (Table 3A) significant TEOAE/ABR relationships were observed, at the 0.05 level, for the ABR threshold variables at 20, 16, 12, and 10 kHz (Fig. 4). A marginally non-significant relationship was observed with the ABR threshold shift at 8 kHz and a non-significant relationship for the click threshold.

Because a visual examination of the marginal relationships between the ABR and TEOAE variables suggested non-linear relationships, we investigated regres-

Table 3
Results from the linear regressions of ABR variables on the TEOAE regressors

ABR threshold shift variables (kHz)	TEOAE regressors	R^2	Adjusted R^2	*P*-value
A				
30	none			
20	•correlation •S/N 4.0 kHz •S/N 5.0 kHz	0.4473	0.3367	0.0272
16	S/N 2.0 kHz	0.3404	0.3016	0.0087
12	S/N 2.0 kHz	0.2133	0.1670	0.0463
10	correlation	0.3100	0.2694	0.0133
8	correlation	0.2039	0.1571	0.0523
click	response	0.1134	0.0641	0.1466
B				
30	Response	0.1612	0.1119	0.0884
20	Correlation	0.2415	0.1969	0.0326
16	Correlation	0.4849	0.4546	0.0009
12	Correlation	0.4246	0.3908	0.0025
10	Correlation	0.4919	0.4620	0.0008
8	•Correlation •S/N 2.0 kHz	0.3948	0.3191	0.0180
Click	•Correlation •S/N 3.0 kHz	0.6920	0.6535	0.0001

The data presented in this table were fitted with first order (linear) models. Probability values higher than 0.05 indicate no significant relationships. (A) Results from post- minus pre-treatment ABR vs. post- minus pre-treatment TEOAE data; (B) results from post-treatment ABR vs. post-treatment TEOAE data. The models showing the TEOAE regressors in a bull-list form, indicate that more than one TEOAE variable was found significantly correlated with the ABR variable.

Table 4
Pearson correlation estimates from the (A) pre-and (B) post-treatment TEOAE variables

	Response	Correlation	S/N 1.5	S/N 2.0	S/N 3.0	S/N 4.0	S/N 5.0
(A) Pre-treatment variables							
Response	1.0	0.35	−0.06	0.16	0.13	0.11	−0.13
Correlation	0.35	1.0	0.22	0.65	0.69	0.11	−0.07
S/N 1.5 kHz	−0.06	0.22	1.0	0.17	0.02	−0.31	0.22
S/N 2.0 kHz	0.16	0.65	0.17	1.0	0.17	0.03	−0.04
S/N 3.0 kHz	0.13	0.69	0.32	0.17	1.0	0.22	0.05
S/N 4.0 kHz	0.11	0.11	−0.32	0.03	0.22	1.0	0.01
S/N 5.0 kHz	−0.13	−0.07	0.22	−0.04	0.05	0.01	1.0
(B) Post-treatment variables							
Response	1.0	0.67	0.48	0.61	0.46	0.57	0.39
Correlation	0.67	1.0	0.58	0.76	0.74	0.75	0.37
S/N 1.5 kHz	0.48	0.58	1.0	0.65	0.60	0.52	0.11
S/N 2.0 kHz	0.61	0.76	0.65	1.0	0.67	0.54	0.19
S/N 3.0 kHz	0.46	0.74	0.60	0.67	1.0	0.58	0.38
S/N 4.0 kHz	0.57	0.75	0.52	0.54	0.58	1.0	0.13
S/N 5.0 kHz	0.39	0.37	0.11	0.19	0.38	0.13	1.0

In (B) all correlation values, except the ones from the last raw referring to S/N ratio at 5 kHz, were found as significant at a 95% confidence interval. The latter is a strong indication that the post-treatment TEOAE responses are less variable.

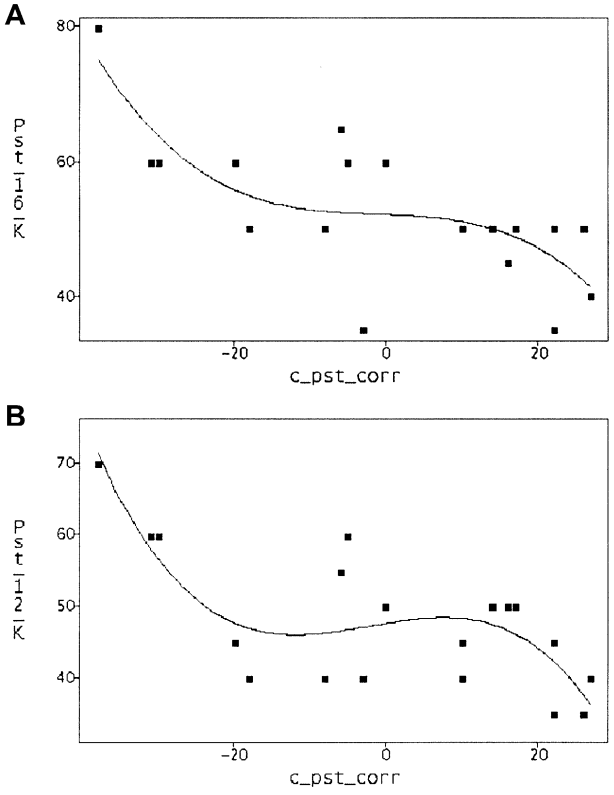

Fig. 4. Data fitting results from a cubic-order regression model, relating the TEOAE correlation values and the ABR post-treatment threshold levels at 16 kHz (panel A) and 12 kHz (panel B). The *x*-axis depicts centered post-treatment TEOAE correlation values (c_pst_corr) and the *y*-axis the post-treatment ABR threshold shift in dB.

sions using non-linear models. Specifically we investigated polynomial models up to order 6, in individual TEOAE variables, and full quadratic models in pairs and triplets of the TEOAE parameters. The best resulting models are summarized in Table A2.

A comparison of Tables 3A and A2 shows that for the post- minus pre-treatment data, the higher order models in Table A2 (almost exclusively three-predictor quadratic models) substantially outperformed the linear models in Table 3A in all measures of fit: R^2, adjusted R^2, and *P*-value, and in the measure of predictive ability, PRESS (see the Appendix for a full description). In these models the TEOAE response, the TEOAE correlation and the S/N at 2.0 kHz appear frequently as predictors. For the corresponding polynomial fits, the proportion of variation in the ABR variables accounted for by the TEOAE variables (measured by R^2) ranged from 0.7126 to 0.8783 (the latter resulting from the ABR threshold level at 10 kHz regressed on a full qua-

Table A1
Bootstrap BCa 95% confidence intervals (Cl) for the difference in correlation values between the pre- and post-treatment TEOAE variables

TEOAE Variable	Correlation	S/N 1.5	S/N 2.0	S/N 3.0	S/N 4.0	S/N 5.0
Response	-1.05, 0.12	-1.11, 0.03	-0.95, 0.10	-1.06, 0.19	-1.13, -0.01	-1.02, 0.01
Correlation	-	-0.88, 0.12	-0.45, 0.10	-0.41, 0.20	-1.05, -0.30	-0.90,0.08
S/N 1.5 kHz	-	-	-0.90, -0.06	-1.17, 0.17	-1.16, -0.38	-0.43,0.66
S/N 2.0 kHz	-	-	-	-1.20, 0.12	-1.01, 0.03	-0.89, 0.30
S/N 3.0 kHz	-	-	-	-	-0.86, 0.21	-0.74, 0.15
S/N 4.0 kHz	-	-	-	-	-	-0.74, 0.77

A difference is considered significant if the corresponding 95% interval does not contain a zero value. The shaded cells correspond to variables showing a significant difference in correlations.

Table A2
Results from the non-linear regressions of ABR variables on the TEOAE regressors

ABR threshold shift variables (kHz)	Type of model	TEOAE predictors	R_2	Adjusted R_2	*P*-value	PRESS
30	3 predictor quadratic	response, S/N 4, S/N 2	0.8010	0.6021	0.250	1873
20	3 predictor quadratic	response, **correlation**, S/N 2	0.8523	0.7045	0.0077	1522
16	3 predictor quadratic	**correlation**, S/N 2, S/N 3	0.8682	0.7363	0.0049	1696
12	3 predictor quadratic	response, **correlation**, S/N 2	0.8346	0.6691	0.0122	2560
	3 predictor quadratic	response, **correlation**, S/N 5	0.7326	0.4652	0.0747	2231
10	3 predictor quadratic	response, **correlation**, S/N 2	0.8783	0.7566	0.0035	765
8	3 predictor quadratic	response, S/N 1.5, S/N 5	0.8330	0.6660	0.0126	1428
Click	3 predictor quadratic	**correlation**, S/N 1.5, S/N 4	0.8121	0.6242	0.0201	2761
	2 predictor quadratic	**correlation**, S/N 3	0.7126	0.6020	0.0032	889

The data were fitted with higher order models as described in Section 2 and the Appendix. Probability values higher than 0.05 indicate no significant relationships. The TEOAE correlation variable, the most often encountered regressor, is presented in bold. (A) Results from post- minus pre-treatment ABR vs. post- minus pre-treatment TEOAE data. (B) Results from post-treatment ABR vs. post-treatment TEOAE data.

dratic model in the TEOAE response, correlation and S/N ratio at 2.0 kHz).

3.3.2. Post-treatment data

For the post-treatment data, the higher-order models presented in Table A3 also substantially outperformed the linear subset models in Table 3B in R^2, adjusted R^2, *P*-value, and PRESS, for all ABR variables except for the click threshold shift values. For that variable, the linear model of Table 3B seems to be a better performer. In addition, for the ABR variables at 20, 16, 12, 10 and 8 kHz, single predictor polynomial models performed better than the multi-predictor quadratic models with respect to at least some of these measures. The proportion of variation in the ABR variables accounted for by the TEOAE variables ranged from 0.2762 to 0.8492. The non-linear relationship between the TEOAE correlation and the ABR threshold shifts at 16 and 12 kHz is reported in Tables A2 and A3.

3.4. On the structure of the post-treatment TEOAE variables

The correlation analysis between the TEOAE variables in the pre- and post-treatment data sets, suggested that the post-treatment TEOAE values were more correlated between them. The intra-variable correlations per data set (pre- and post-treatment) are shown in Table 4. It should be noted that only two significant correlations were found in the pre-treatment TEOAE data set (Table 4A), between the TEOAE correlation and the S/N ratio at 2.0 and 3.0 kHz respectively.

The differences in the correlation values of the pre- and the post-treatment sets were evaluated with bootstrap BCa confidence intervals (see the Appendix). The results, shown in Table A1 in the Appendix, indicate that for only two variables (S/N 2.0 kHz and S/N 4.0 kHz) the observed correlation differences are significant at the 0.05 level.

Table A3
Results from the non-linear regressions of ABR variables on the TEOAE regressors from post-treatment ABR vs. post-treatment TEOAE data

ABR threshold shift variables (kHz)	Type of model	TEOAE predictors	R_2	Adjusted R_2	*P*-value	PRESS
30	3 predictor quadratic	S/N 1.5, S/N 4, S/N 5	0.8492	0.6985	0.0084	1311
20	3 predictor quadratic	**correlation** S/N 1.5, S/N 5	0.7701	0.5403	0.0431	3355
	cubic	**correlation**	0.4240	0.3087	0.0362	1387
16	quartic	**correlation**	0.6026	0.4891	0.0082	1412
	cubic	**correlation**	0.5644	0.4773	0.0050	1530
12	3 predictor quadratic	**correlation**, S/N 4, S/N 5	0.8006	0.6013	0.0252	4233
	cubic	**correlation**	0.6324	0.5589	0.0015	895
10	quintic	**correlation**	0.7200	0.6122	0.0027	1456
	cubic	**correlation**	0.6423	0.5708	0.0012	856
8	cubic	**correlation**	0.6347	0.5617	0.0014	910
Click	quartic	**correlation**	0.7043	0.6198	0.0012	884
	cubic	**correlation**	0.6347	0.5617	0.0014	910

The data follow the format of Table A2.

4. Discussion

According to a number of studies in the literature (Hotz et al., 1994; Arruda et al., 1996; Meech et al., 1998;) the TEOAEs are considered good indicators of ototoxic effects induced by salicylate and aminoglycosides. The present study presents evidence suggesting that the TEOAEs can be used successfully to detect ototoxic effects induced by the antineoplastic drug cisplatin. The Sprague–Dawley rats presented an excellent survival rate 72 h after the high-dose cisplatin administration, a performance probably aided by the daily oral hydration of the animals with saline solution.

The first objective of the study was the verification of the monitoring capacity of the TEOAEs for the possible ototoxic effects of cisplatin. The data from the post-treatment responses suggest that cisplatin significantly alters the cochlear function as recorded by the TEOAEs. Significant changes across all the TEOAE variables have been observed, suggesting that the TEOAEs have a good potential as descriptors of the cisplatin-induced ototoxicity in general.

The second objective of the study was the identification of a subset of TEOAE variables to be used in the cisplatin monitoring. The presented data show that the most significant alterations were observed in the TEOAE response, TEOAE S/N ratio at 5.0 kHz and TEOAE correlation values. These findings are in accordance with the data reported by previous studies (Hatzopoulos et al., 2000; Sochalingam et al., 2000).

In terms of the observed ABR threshold shifts, the responses evoked by high-frequency stimuli resulted as the most affected, showing the highest mean threshold shift (35.57 dB at 30 kHz). The threshold shift values reported in this study match the data presented by Campbell et al. (1996) and Ravi et al. (1995).

The third objective of the study was the evaluation of the relationship between TEOAE and ABR variables. This relationship has been sought in order to add prognostic utility to the TEOAE monitoring, in terms of knowledge of the functional status of various cochlear partitions resonating at mid to high frequencies (the ototoxic effects of cisplatin manifest first at basal cochlear regions corresponding to the high frequencies). For this purpose we have formulated the hypothesis that the most sensitive TEOAE variable (TEOAE response) should correlate well with the ABR responses (and the corresponding electrophysiological hearing thresholds) evoked by tone pips of 8–30 kHz, in order that the TEOAE-based protocols could have a good prognostic potential.

Recent evidence from studies on cochlear modeling and the TEOAE structure have provided good arguments favoring the validity of the proposed hypothesis, such as; (A) the TEOAE response of the rat is recorded within an initial 1.0–19.5 ms window and it is possible that a portion of the high-frequency information (from basal generators) is preserved in the recorded TEOAE response. The limiting factor in this case is the sampling frequency of the ILO, resulting in an upper bandwidth limit of 12.5 kHz; (B) according to a number of cochlear TEOAE simulation models (Zweig and Shera, 1995; Shera and Guinan, 1999) the basilar membrane reflections are re-reflected at the stapes towards the apical portion of the cochlea, thus stimulating other cochlear partitions of frequencies different (and probably lower) than the resonating frequency of the original reflection site (OAE generator). In this context, the TEOAE information originated at a higher frequency can be mapped at a lower frequency of the TEOAE response. Additional support for this hypothesis comes from recent studies of Withnell and Yates (1998) and Withnell et al. (1998). These studies have presented evidence according to which when the cochlea is stimulated by high frequency stimuli, the TEOAE responses are enhanced at low frequencies due to the generation of intermodulation distortion products, which interact with the basilar membrane motion, increasing it's vibration at lower frequencies. In this context, if we assume that the acoustic click stimulus might stimulate high frequency cochlear partitions, then according to the hypotheses presented the energy from the high frequency TEOAE generators might be manifested as energy at lower TEOAE frequencies.

The presented data suggest that while there is a significant linear component to the prediction of ABR values by TEOAE values (for both post-treatment and post- minus pre-treatment data sets), non-linear models relating the two, show a substantially stronger relation in almost every case. For the post-treatment data, significant relationships have been observed for the TEOAE correlation and the ABR threshold values for clicks, and tone pips at 10.0, 12.0, 16.0 and 20.0 kHz. The results support the hypothesis that the TEOAEs can be used to predict functional cochlear changes located even at higher frequencies than the ones present in the response spectrum. The most sensitive TEOAE variable to the ototoxic effects (the TEOAE response) did not correlate well with the ABR threshold shifts in the non-linear models for post-treatment data, but did in the non-linear models for the differenced (i.e. post- minus pre-treatment) data set. The best performance in terms of relating ABR and TEOAE variables was observed in the TEOAE correlation. In this context, it can be said that the alteration of the TEOAE correlation value in a rat animal model, due to the cisplatin administration, includes information from various cochlear segments which resonate as high as 16–20 kHz.

A series of analyses which provided more information on the structure of the TEOAE recordings after the cisplatin treatment were performed and have led to the formulation of the following hypothesis. It could be postulated that the post-treatment TEOAE variables reflect the activity of a few common cochlear TEOAE generators. This hypothesis assumes that the cisplatin administration has inhibited/damaged the functionality of various cochlear segments, which contribute to the more individualistic content, per frequency band, of the TEOAE 'response'. A possible mechanism for such an effect is the shutting down of the metabolic resources of groups of OHCs (emission generators). Data in the literature which relate the ototoxic effects of cisplatin with alterations of the stria vascularis (Campbell et al., 1996; Meech et al., 1998) are in support of this hypothesis.

One limitation with the use of TEOAEs in detecting cisplatin ototoxicity is that the test only reveals one aspect of cisplatin ototoxicity, namely the toxic effect on the cochlear micro-mechanics related to the OHCs. Although the TEOAE correlation values reflect changes of the cochlear function in a number of frequencies (higher than the stimulus bandwidth), the exact frequency relationship between the observed threshold shifts and the TEOAEs remains undefined and additional studies are needed to elucidate this aspect.

5. Summary

The Sprague–Dawley rat animal model, treated with 16 mg/kg of cisplatin in a 30 min slow intra-peritoneal infusion, showed excellent survival rate 72 h after treatment. All treated animals demonstrated significant shifts of the ABR threshold and TEOAE amplitude levels. The Sprague–Dawley rat animal model proved to be adequate for cisplatin monitoring studies. Our findings can be summarized in the following statements:

1. Cisplatin-induced ototoxic effects can be detected efficiently and accurately by TEOAE recordings. The post-treatment TEOAE responses are characterized by smaller signal amplitudes and lower signal variability.
2. The relationship between the ABR and TEOAE variables was found to be non-linear and the best relationships were obtained by cubic and quartic models. For the majority of the post-treatment ABR variables, the 'TEOAE correlation' was a significant regressor.
3. The most sensitive indicator of ototoxicity among the TEOAE variables was shown to be the TEOAE response which did not correlate well with the ABR threshold shifts in the non-linear models for post-treatment data, but did in the non-linear models for post- minus pre-treatment data.
4. The TEOAE 'correlation' can be used as an efficient descriptor of ototoxic insults of the cochlear function. It was shown that this variable is significantly correlated with the ABR thresholds shifts at the frequencies 10, 12 and 16 kHz.

6. Notes

The TEOAE visualization software used in the study was developed by a scientific collaboration between the technical University of Warsaw, Poland, and the Department of Audiology of Ferrara University, Italy. The viewer uses the data already stored by the ILO software in the dta ILO files. The program can be downloaded for free, from the Otoacoustic Emissions Portal web address http//www.otoemissions.org.

Appendix

1. Bootstrap estimation of post–pre correlation differences

The bootstrap procedure is a distribution-free resampling method which can produce confidence intervals in situations where the standard theory does not give good results: either the data do not satisfy the usual assumptions (such as normality), or it is necessary to use a non-standard estimator about which little theory exists. The basic idea behind this procedure is to approximate the sampling distribution of an estimator with the distribution of estimates computed from a set of bootstrap samples, each of the same size as the original data. The observations in each bootstrap sample are selected randomly and independently with replacement from the original data. The endpoints of a naive level (L) confidence interval can be obtained as the $(1\text{-}L)/2$ and $(1+L)/2$ quantiles of the distribution of bootstrap estimates. We use an improved version, the BCa confidence interval, which adjusts the quantiles defining the endpoints of the confidence interval to account for bias and non-constant standard error of the estimator. More information on the procedure can be found in chapter 14 of Efron and Tibshirani (1993).

In calculating the bootstrap intervals for the difference of each pair of before–after TEOAE Pearson correlations, 2000 bootstrap samples were used. The estimator was the difference between the sample Pearson correlation of the post-cisplatin variables and the

sample Pearson correlation of the pre-cisplatin variables.

2. Regressions of ABR variables on TEOAE regressors: non-linear models

The following fitting rules were used:

1. Two kinds of models were used: (a) polynomials in a single predictor of order up to 5, and (b) quadratic polynomial models in two and three predictors. The two predictor models in (b) are of the form $y = b_0 + b_1 \times_1 + b_2 \times_2 + b_{12} \times_1 \times_2 + b_{11} \times_1^2 + b_{22} \times_2^2$. The three predictor models in (b) are of the form $y = b_0 + b_1 \times_1 + b_2 \times_2 + b_3 \times_3 + b_{12} \times_1 \times_2 + b_{13} \times_1 \times_3 + b_{23} \times_2 \times_3 + b_{11} \times_1^2 + b_{22} \times_2^2 + b_{33} \times_3^2$.
2. In each model all terms were included: subset models were not considered except those of lower order in (a).

In order to select the 'best' models from among those in 1 (a) and (b), two measures were used:

1. Adjusted R^2. This is basically the R^2 estimate adjusted for the number of regressors. Using R^2 as a criterion tends to result in overfitting, as adding a regressor always increases it. However, if the added regressor does not increase R^2 sufficiently, the adjusted R^2 will decrease. A larger adjusted R^2 is better.
2. PRESS (PREdictive Sum of Squares) estimate. PRESS measures the predictive power of the model. PRESS is given by the formula:

$$PRESS = \sum_{i=1}^{n} (y_i - \hat{y}_{(i)})^2$$

where y_i is the response from animal i and $\hat{y}_{(i)}$ is the predicted value of that response computed from the data set with animal i removed. Unlike adjusted R^2, PRESS does not take into account the number of regressors in the model, but it (a) gives some measure of the predictive power of the model, and (b) helps detecting overfitting (if the model is too strongly reliant on a particular observation, the model fit to the rest of the data will not predict that observation well, and PRESS will be large.)

It should be noted that a number of models in Tables A2 and A3, which show low R^2 values, were included because they presented lower PRESS values than other models for the same ABR variable, which may indicate that the high R^2 values of the latter are due to overfitting, a common problem with small data sets. Complete statistical details are shown in Table 4.

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