

ORIGINAL PAPER

Noise-induced hearing loss: a study on the pharmacological protection in the Sprague Dawley rat with N-acetyl-cysteine

Ipoacusia da rumore: studio sulla protezione farmacologica nel ratto Sprague Dawley con N-acetil-cisteina

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Key words

Hearing loss • Noise • Corti's organ hair cells • Otoacoustic emissions • N-acetyl-cysteine • Sprague Dawley Rats

Parole chiave

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Summary

Noise-induced hearing loss is one of the most common causes of deafness and, at present, there is no treatment for the recovery of the normal hearing threshold after prolonged exposure to loud acoustic stimuli and the generation of acoustic trauma. Prolonged exposure to noise can cause oxidative stress in the cochlea which results in the loss (via apoptotic pathways) of the outer hair cells of the organ of Corti. It has been demonstrated that some antioxidant molecules, for example L-N-acetyl-cysteine, can prevent oxidative stress in the inner ear. Aim of the study was to evaluate whether L-N-acetyl-cysteine, given at various dosages, can preserve the fine structures of the cochlea from the insult of continuous noise. A series of 18 Sprague Dawley male albino rats were exposed to continuous noise (8 kHz octave band noise, 105 dB SPL, 4 hours), and cochlear functionality was evaluated by recordings of transient evoked otoacoustic emissions and distortion products otoacoustic emissions). The group which showed the best protection was that which received a total dosage of 1500 mg/kg of L-N-acetyl-cysteine. These data suggest that while L-N-acetyl-cysteine can partially protect the cochlea from continuous noise, the protection effect is strongly dose-dependent: lower dosages do not fully protect the cochlea and higher dosages can damage the rat systemically (e.g. pulmonary toxicity).

Riassunto

L'ipoacusia da rumore è una delle cause più comuni della perdita di udito, ed attualmente non esiste nessuna cura che possa conferire recupero uditivo dopo il trauma acustico. Il rumore continuo causa la perdita per apoptosi delle cellule cigliate esterne dell'organo del Corti. È stato dimostrato che degli antiossidanti come la N-acetil-cisteina possono proteggere l'orecchio interno dallo stress ossidativo. Questo studio si occupa di capire se la N-acetil-cisteina, somministrata a varie dosi, possa proteggere le strutture fini della coclea dalla esposizione al rumore continuo. Sono stati esposti a rumore continuo (centrato a 8 kHz, 105 dB SPL, 4 ore) 18 ratti albini maschi Sprague Dawley, la cui funzionalità cocleare è stata valutata mediante registrazioni delle emissioni otoacustiche evocate da un transiente (TEOAE) e registrazioni delle emissioni otoacustiche dai prodotti di distorsione (DPOAE). Gli animali esposti a rumore continuo che hanno mostrato la protezione migliore sono stati quelli che hanno ricevuto una dose totale di 1.500 mg/kg di N-acetil-cisteina. Questi risultati suggeriscono che la N-acetil-cisteina può proteggere parzialmente la coclea dal rumore continuo, un ulteriore dato che è emerso dai dati sperimentali è che la protezione è fortemente dose-dipendente: i dosaggi più bassi non proteggono la coclea completamente e i dosaggi più alti possono creare un danno sistemico nel ratto (es. tossicità polmonare).

Introduction

The most frequent causes of hearing loss are: age, genetic factors, drug ototoxicity and acoustic trauma. The cellular mechanisms which lead to noise-induced hearing loss (NIHL) are not yet clear. Exposure to continuous high intensity noise initiates a cascade of reactions at a cochlear level, leading to death

by apoptosis of the outer hair cell (OHC) of the organ of Corti organ¹. The reactive oxygen species (ROS) play a primary role in NIHL and their cellular concentrations are the starting apoptotic signal in the OHC. Ohlemiller et al.² have demonstrated that in the mouse cochlea, the level of hydroxy-radicals is increased up to ten times after exposure to continuous noise. Data from other studies^{3,4} have also shown

that the level of ROS is, on average, four times higher in the exposed than the control non-exposed animals. More recently Ohinata et al.⁵ have shown that the levels of 8-isoprostane (a ROS concentration tracer) were 30 times higher in noise-exposed guinea pigs (4 kHz, 115 dB SPL, 5 hr) than in the control group. Data from human studies and NIHL, in factories where the average level of environmental noise was between 95 to 110 dB SPL, have indicated that the tested subjects had unusually high levels of ROS, a fact which was explained as the result of prolonged exposure to noise⁶.

The role of antioxidants in the protection of the inner ear from environmental noise, has been widely studied recently. Ohinata et al.^{5,7,8} have shown that in the guinea pig, glutathione (GSH) protects the inner ear from ROS damage induced by noise. Vitamin E (a well-known antioxidant) has been used, with good results, to prevent NIHL in laboratory animals⁹.

At present, the interest of the scientific community is focused in the direction of developing new drugs which can fully protect the inner ear and the neural fibres from noise damage. This issue is justified by the fact that, in many noisy environments, the workers still develop NIHL, despite the fact that they use mechanical noise-protection. Since the sound energy of noise is distributed almost equally to both ears, the protector-drug can be given systemically (i.e., orally). Numerous studies have approached the local administration (i.e., trans-tympanic infusion) of noise-protectors, but such a scenario cannot be part of a preventive policy against exposure to noise.

Our experimental design evaluated the protective effects of L-N-acetyl-cysteine (L-NAC) in a Sprague-Dawley rat animal model. From the two possible modes of experimental design (dosage and time), we chose to explore the efficacy of the dosage-dependence of the drug. The initial choice of L-NAC was based on the fact that: (i) it is directly involved in the neutralization reactions of the superoxide (the principal apoptotic starter), and (ii) it is the most effective and the most immediate forerunner of GSH. The cochlear function of the animals was evaluated by recordings of otoacoustic emissions (OAE) and auditory evoked potentials (used as the gold standard). The OAE recordings have been widely used in the past, by the same Authors, to evaluate the damaging effects on the cochlea not only from noise but also from cisplatin¹⁰, and various anaesthesia agents¹¹.

Methods

ANIMALS AND DRUGS

A total of 18 male albino Sprague Dawley rats (from Charles River, Italy) were used in the study (mean weight of the animals 250 ± 15 g). The animals were

divided into 5 groups: group A (n = 4), B (n = 4), C (n = 4), D (n = 2) and E (n = 4, control). L-NAC (Sigma, St. Louis, USA) was administered intra-peritoneally (i.p.), at various dosages, after being diluted in saline at a concentration of 80 mg/ml/animals. The drug was administered 6 times within the time span of the experiments (Fig. 1). Group A received a total of 1200 mg/kg, group B a total of 1500 mg/kg, group C a total of 1800 mg/kg, and group D a total of 2100 mg/kg. The animals were anaesthetized before each recording session by a ketamine-xylazine cocktail.

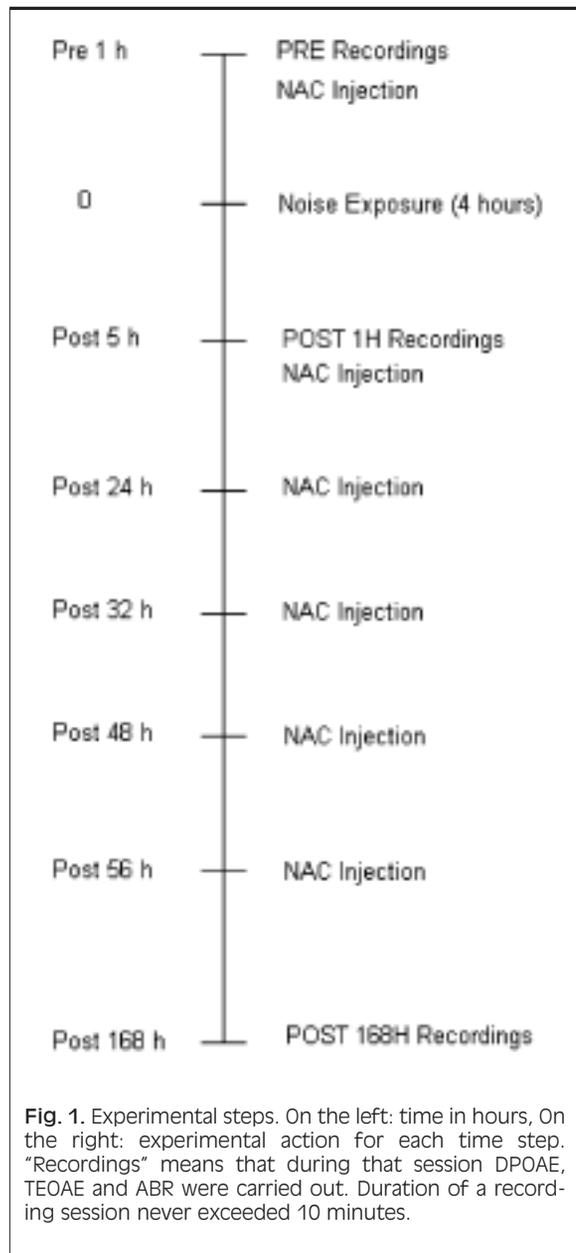


Fig. 1. Experimental steps. On the left: time in hours, On the right: experimental action for each time step. "Recordings" means that during that session DPOAE, TEOAE and ABR were carried out. Duration of a recording session never exceeded 10 minutes.

Each ml of anaesthesia contained 0.33 ml of 2% xylazine (Rompun; Bayer, Leverkusen, Germany), 0.33 ml of 10% ketamine (Ketavet 100x, Intervet, Aprilia, Italy) and 0.33 ml of saline 0.9% (Eurospital, Trieste, Italy).

ELECTROPHYSIOLOGICAL RECORDINGS

During all measurements (OAE and ABR), the body temperature of the animal was maintained at 37 ± 0.5 °C by means of a temperature control device (Harvard Apparatus, Holliston, MA, USA). A rectal probe was introduced in order to assess changes in body temperature, and a homeothermic blanket, under the rat's body, regulated the heating in order to maintain body temperature constant throughout the time needed for the acquisition of recordings.

Three sets of data were collected: 1 hour before (PRE), 1 hour after (POST 1 H), and 7 days (POST 168 H) after the noise exposure.

Otoacoustic emissions

The recordings of the transient evoked otoacoustic emissions (TEOAE) were conducted in a soundproof chamber by a ILO 92 (Otodynamics Ltd, Herts, UK) device. The TEOAE responses were evoked by non-linear 80 clicks with an amplitude of 63 ± 2 dB p.e. SPL, according to the standard ILO non-linear protocol¹². To avoid excessive stimulus artefacts TEOAEs were analyzed in the interval from 1 to 6 ms (applying a window function to the original 20 ms recording). For each response, estimates of reproducibility (Repro) and signal to noise ratios at 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0 kHz were calculated. To optimize the amplitude of the TEOAE response, the anaesthetized animal was placed under a stereotaxic device which held a neonatal ILO probe. The latter was connected to the right external acoustic meatus with a little tube (35 mm in length).

The recordings of the distortion product otoacoustic emissions (DPOAE) were conducted in the same soundproof chamber, as the TEOAE, by a Starkey 2000 (Starkey Labs, Eden Prairie, MN, USA) device. The DPOAE amplitudes were analyzed at the frequencies from 4.0 to 16.0 kHz (referred to f₂) and, for each octave, 12 frequency points were sampled. The frequency ratio between primaries was fixed to 1.21. Each recording was made on the average from 4 s of data sampling, and the noise tolerance was fixed at -15 dB SPL. The recordings were elicited by asymmetrical DPOAE protocols (L1 > L2), at three different stimulus intensities. Asymmetrical protocols are generally considered a better choice to identify cochlear dysfunction^{13,14}. According to the eliciting stimuli, the protocols were defined as: 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). All measurements were conducted on the right ear of each tested animal.

Auditory Brainstem Responses (ABR)

The ABR responses were recorded by 3 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 the sound treated chamber where OAE were recorded. The sound transducer, a Motorola weeter (flat response ± 1.5 dB from 4.0 to 35 kHz), was placed 4 cm away the rat's ear.

The ABR were amplified 20000 times and filtered from 20 to 5000 Hz. Each recording was the average of 1000 individual responses. The ABR were generated in response to 100 μ s alternated clicks and 8, 10, 12, 16 tone pips (1 ms rise-fall time, 10 ms plateau), in the range 100 to 40 dB SPL. The sound intensity of the stimulus was varied in 5 dB intervals. Threshold was based on the visibility and reproducibility of wave III, and at the minimum threshold level two recordings were acquired. Threshold was defined as the lowest intensity at which a measurable ABR wave was seen in two averaged runs. As in previous studies^{10,11}, the threshold level of the SD rat, at frequencies up to 16 kHz, was found to be 40 dB SPL. Ear plugs were used to occlude the contra-lateral ear in order to avoid binaural stimulation at high stimulus intensities (100 dB SPL).

NOISE EXPOSURE

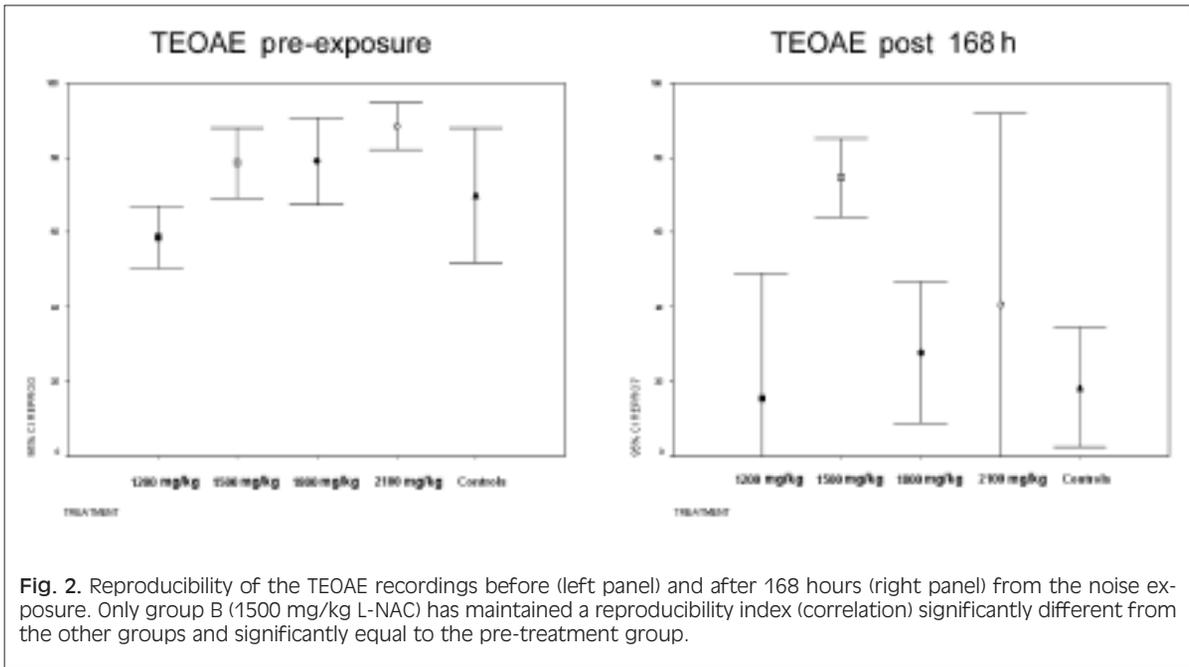
The animals were exposed to 105 dB SPL, 8 kHz OB (octave band) noise. The exposure to noise was facilitated by 4 tweeter speakers placed on the top of a 1 m³ box. The total duration of noise (no pauses) was 4 hours. During this time, the animals were free to eat and drink (inside the appropriate animal containers).

STATISTICAL ANALYSIS

Paired t-tests and analyses of variance (ANOVA) were used to compare the performance between the different groups and at the 3 experimental times (PRE, POST 1 H, POST 168 H). For all analyses, a significance level of 0.5 was used.

TIME ORDER OF THE EXPERIMENTS

The methodological order and time of the conducted experiments (i.e., OAE measurements, L-NAC injections and acoustic trauma generation) are outlined in Figure 1. Data were collected in this order: DPOAEs, TEOAEs and then ABR. The total duration of each recording session never exceeded 10 min.



Results

The analysis of the responses from the PRE exposure data set indicated that: (i) the pre-exposure recordings were normal for each animal tested; and (ii) that pre-exposure recordings between the experimental groups (i.e., A, B, C, D, E) were not significantly different.

For the POST 1 H data set, the ABR, DPOAE and TEOAE data indicated significant differences with the pre-exposure data set, which was interpreted as an indication of cochlear damage due to noise exposure (data not shown).

The TEOAE data analysis (paired t-tests on S/N ratios and ANOVA analysis on reproducibility) from data set POST 168 H indicated that the only group which differed significantly from group E (controls, non-treated animals) was group B which had received a total of 1500 mg/kg L-NAC. The other experimental groups (A, C and D) did not present any statistically significant differences (i.e., no protection was observed) with the control animals of Group E. The findings are summarized in Figure 2 and Table I showing the TEOAE reproducibility, good global index of OHC activity according to across the tested groups, at time PRE and POST 168 H¹¹.

The paired t-test analysis of the DPOAE recordings suggested that the cochlear function of the exposed animals was altered and significant differences were observed in the S/N ratios of 8 out of the 12 tested frequencies. Good DPGRAM responses have been

observed only for the higher intensity protocols (P2 and P3). For the P1 protocol, the recordings did not show any significant protection even for the 1500 mg/kg dosage (Fig. 3).

The left panels of Figure 3 represent the pre-recordings for the three experimental protocols. The data indicate that there are no significant differences between the DPOAE-magnitudes from the three tested protocols. The responses obtained by the P1 protocol present, as expected, lower amplitudes which may be explained by the fact that a lower stimulus energy is reaching the cochlear partition. The right panels of Figure 3 show the recordings obtained 168 hours after the noise exposure. The data indicate that only group B animals (1500 mg/kg NAC) presented acceptable DPOAEs (in terms of S/N ratios). The animals in groups A, C, D and E (controls) showed DPOAE responses which were below the level of the noise floor, hence the responses were considered as not present.

In terms of ABR threshold shift, the data from group B (POST 168 H) presented the following shifts in dB: at 8 kHz 20 dB, at 10 kHz 15 dB, at 12 kHz 12 dB and at 16 kHz 8 dB. From earlier experiments on ototoxicity evaluation, on the SD rat model^{10,11}, it was known that threshold shifts > 40 dB would indicate border-line to significant hair cell losses. In this context, the ABR data from the POST 168 H were interpreted as almost normative and reflecting a partial protection efficacy of the 1500 mg/kg dosage of L-NAC.

Table I. ANOVA analysis of the reproducibility (signal correlation) of the TEOAE recording post 168 hours. The recordings from group B (1500 mg/kg L-NAC) differ significantly (i.e. show better values) from the other groups.

Dependent variable	(I) TREAT.	(J) TREAT.	Mean difference (I-J)	SE	p	95%CI	
						Lower Bound	Upper Bound
REPRO 168 H	1200	1500	-69.6667*	7.8577	.019	-119.3098	-20.0235
		1800	-12.4167	7.8577	.855	-59.9395	35.1062
		2100	-25.6667	9.3918	.273	-76.9281	25.5948
		CON.	-2.9167	7.8577	1.000	-47.9323	42.0990
	1500	1200	69.6667*	7.8577	.019	20.0235	119.3098
		1500	57.2500*	7.2749	.003	27.7753	86.7247
		2100	44.0000	8.9098	.052	^a	
		CON.	66.7500*	7.2749	.001	40.6187	92.8813
	1800	1200	12.4167	7.8577	.855	-35.1062	59.9395
		1500	-57.2500*	7.2749	.003	-86.7247	-27.7753
		2100	-13.2500	8.9098	-.576	-47.8378	21.3378
		CON.	9.5000	7.2749	.884	-21.8312	40.8312
	2100	1200	25.6667	9.3918	.273	-25.5948	76.9281
		1500	-44.0000	8.9098	.052	^a	
		1800	13.2500	8.9098	.576	-21.3378	47.8378
		CON.	22.7500	8.9098	.134	-8.3032	53.8032
	CON	1200	2.9167	7.8577	1.000	-42.0990	47.9323
		1500	-66.7500*	7.2749	.001	-92.8813	-40.6187
		1800	-9.5000	7.2749	.884	-40.8312	21.8312
		2100	-22.7500	8.9098	.134	-53.8032	8.3032

* The mean difference is significant at the .05 level; ^a: range values cannot be computed

Discussion

The aim of the study was to evaluate the protective effects of L-NAC, at different administration dosages, in a SD rat model for which we have adequate information on drug induced toxicity. Evaluations were performed on 4 drug dosages, corresponding to 1200, 1500, 1800 and 2100 mg/kg.

Data obtained suggest that only one concentration (1500 mg/kg) partially protects inner ear cochlear function, while the other concentrations (low or high) do not appear to be of protective value. In this context, and judging from the variability of the data, it might be postulated that it is difficult to predict a trans-species protective effect of L-NAC. The latter is a difficult drug to dose in the case of NHIL: at low dosages, it does not offer efficient protection, whereas, at high dosages, it is not only ototoxic but also systemically toxic¹⁵. Behavioural data on the well-being of animals in groups C and D have indicated that doses > 1500 mg/kg generate undesirable side-effects. The main signs of untoward effects were displayed by changes in colour and appearance of the animal's fur, and changes in the mode of movement and equilibrium. Similar conclusions

were reached in another study by Duan et al.¹⁵ where Sprague Dawley rats were exposed to impulsive noise. Untoward side-effects were reported including lung toxicity as the main reason for the onset of associated disorders.

Data from the ABR recordings confirmed the data from the OAE responses and indicated that at the tested dosages L-NAC partially protects the inner ear from noise damage. Since the data suggest that the effect of protection is dosage dependent, it might be hypothesized that a lower dosage protocol (i.e., fewer drug administrations than the 6 used here) might offer a more efficacious inner ear protection. In this context, dosage variability becomes time-variability. The proper timing issue might explain why the 1200 and 1800 mg/kg dosages did not protect the outer hair cells efficiently. ROS is a time-dependent phenomenon (peaks within 12 hours from exposure to noise administration according to Duan et al.¹⁵). The toxicity of L-NAC is probably related to dosages administered when the ROS peak is rapidly decreasing (i.e., after T = 12 hours). In accordance with the tested experimental protocol, L-NAC was administered also at times T = 24, 32, 48 and 56 H. The 1800 mg/kg dosage overloaded the OHCs because, at the

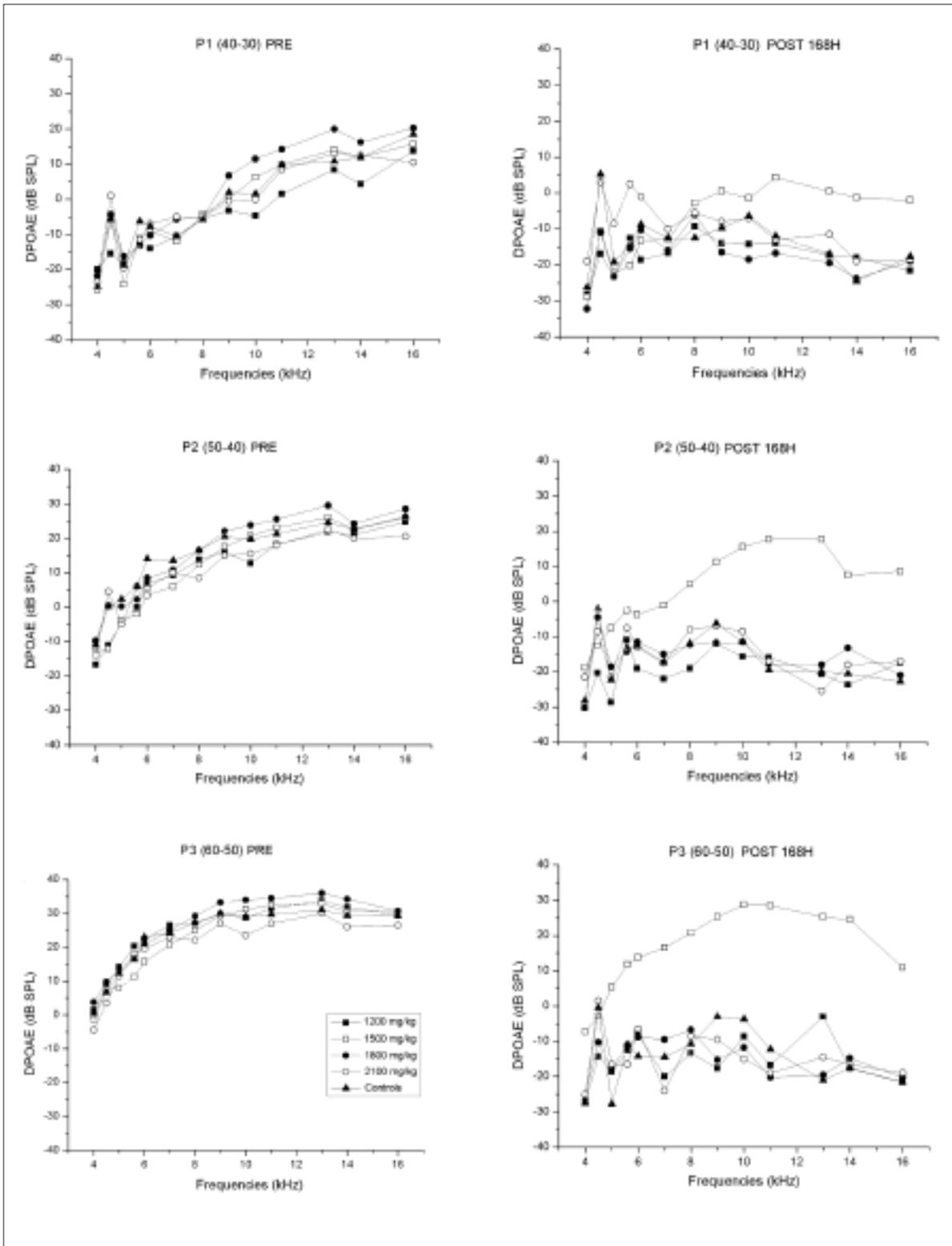


Fig. 3. DPOAE recordings before (left) and after 168 hours (right) exposure to noise. Only group B (1500 mg/kg L-NAC) shows significant differences from the other groups in the recordings POST 168 H for the P2 and P3 protocols.

last 3 L-NAC administration times, the ROS production was significantly decreased. The 1200 mg dosage did not protect because the quantity of L-NAC did not counteract, the loss of GSH within the time window of 12 h.

In this context, we can conclude that an increase in the amount of L-NAC administered does not produce additional protective effects on the OHCs and that 6 administrations of L-NAC are probably not necessary. We postulate that it would be advantageous to test the time-dependency of the drug with fewer or even with a single L-NAC administration. If the administration of L-NAC takes places before T = 12 H, it is quite possible that even higher single dosages (i.e., > 350 mg/kg) could be tolerated, could provide more OHC protection and be less toxic systemically. The proposed approach should be controlled at 168 or even 336 h to verify that (i) the tested subjects do

not present significant inner ear alterations (OAE data); (ii) the neural pathways are not affected (ABR data).

Since the main aim of this line of research is the prevention of inner ear damage from accidental or prolonged exposure to noise (pilots, industrial personnel etc.), in the above scenario, one should also consider the possibility to intervene locally (administration of the anti-oxidant closer to the inner ear). Local administration of protector molecules has shown excellent results^{1,2,8} but this method is not favoured clinically due to the invasiveness of the procedures tested on various animal models. Newer non-invasive techniques (i.e., use of a gel-vehicle), might offer better and fuller protection from noise in real clinical scenarios, but the dose-response relationship of the protector drugs, encapsulated in the vehicle gels, have to be precisely determined.

References

- ¹ Hu BH, Henderson D, Nicotera TM. *Involvement of apoptosis in progression of cochlear lesion following exposure to intense noise*. *Hear Res* 2002;166:62-71.
- ² Ohlemiller KK, McFadden SL, Ding DL, Flood DG, Reaume AG, Hoffman EK, et al. *Targeted deletion of the cytosolic Cu/Zn-superoxide dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss*. *Audiol Neurootol* 1999;4:237-46.
- ³ Ohlemiller KK, Wright JS, Dugan LL. *Early elevation of cochlear reactive oxygen species following noise exposure*. *Audiol Neurootol* 1999;4:229-36.
- ⁴ Ohlemiller KK, Dugan LL. *Elevation of reactive oxygen species following ischemia-reperfusion in mouse cochlea observed in vivo*. *Audiol Neurootol* 1999;4:219-28.
- ⁵ Ohinata Y, Yamasoba T, Schacht J, Miller JM. *Glutathione limits noise-induced hearing loss*. *Hear Res* 2000;146:28-34.
- ⁶ Kaygusuz I, Ozturk A, Ustundag B, Yalcin S. *Role of free oxygen radicals in noise-related hearing impairment*. *Hear Res* 2001;162:43-7.
- ⁷ Ohinata H, Saha SK, Ohno T, Hata N, Misawa Y, Kuroshima A. *Effect of dietary docosahexaenoic acid on in vitro thermogenesis and fatty acid compositions of brown adipose tissue*. *Jpn J Physiol* 1998;48:189-96.
- ⁸ Ohinata Y, Miller JM, Altschuler RA, Schacht J. *Intense noise induces formation of vasoactive lipid peroxidation products in the cochlea*. *Brain Res* 2000;878:163-73.
- ⁹ Hou F, Wang S, Zhai S, Hu Y, Yang W, He L. *Effects of alpha-tocopherol on noise-induced hearing loss in guinea pigs*. *Hear Res* 2003;179:1-8.
- ¹⁰ Hatzopoulos S, Di Stefano M, Campbell KC, Falgione D, Ricci D, Rosignoli M, et al. *Cisplatin ototoxicity in the Sprague Dawley rat evaluated by distortion product otoacoustic emissions*. *Audiology* 2001;40:253-64.
- ¹¹ Hatzopoulos S, Petruccielli J, Laurell G, Finesso M, Martini A. *Evaluation of anesthesia effects in a rat animal model using otoacoustic emission protocols*. *Hear Res* 2002;170:12-21.
- ¹² Kemp DT, Ryan S, Bray P. *A guide to the effective use of otoacoustic emissions*. *Ear Hear* 1990;11:93-105.
- ¹³ Whitehead ML, Lonsbury-Martin BL, Martin GK. *Evidence for two discrete sources of 2f1-f2 distortion-product otoacoustic emission in rabbit. II: Differential physiological vulnerability*. *J Acoust Soc Am* 1992;92:2662-82.
- ¹⁴ Whitehead ML, McCoy MJ, Lonsbury-Martin BL, Martin GK. *Dependence of distortion-product otoacoustic emissions on primary levels in normal and impaired ears. I. Effects of decreasing L2 below L1*. *J Acoust Soc Am* 1995;97:2346-58.
- ¹⁵ Duan M, Qiu J, Laurell G, Olofsson A, Counter SA, Borg E. *Dose and time-dependent protection of the antioxidant N-L-acetylcysteine against impulse noise trauma*. *Hear Res* 2004;192:1-9.

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