Otology

Protective effects of N-acetylcysteine on noise-induced hearing loss in guinea pigs

Effetti protettivi della N-acetilcisteina nel modello animale dell'ipoacusia da rumore

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SUMMARY

Increasing evidence suggests the involvement of oxidative stress in noise-induced hearing loss. The present study analysed, in an animal experimental model, the time course of the pathogenic mechanisms of noise-induced cochlear damage and the efficacy of the antioxidant drug N-acetylcysteine in reducing noise ototoxicity. Animals were divided into two groups, exposed to noise one treated with N-acetylcysteine for 3 days and one (the control group) with saline. Acoustic trauma was induced by a continuous pure tone of 6 kHz, at 120 dB SPL for 30 minutes. Electrocochleographic recordings were made from an implanted round window electrode and the compound action potentials were measured daily at 2-16 kHz for 7 days. Morphological changes were analysed by scanning electron microscopy. The acoustic threshold measured 1 hour after acoustic trauma was elevated in the control group to 70-90 dB in the higher frequencies of the compound action potential audiogram, with a maximum threshold elevation ranging between 12 and 16 kHz. During the first 24 h, following acoustic trauma, there was a partial recovery of compound action potential thresholds of about 20 dB to reach a final threshold elevation of about 50-70 dB; there was no further improvement over the remaining experimental week. Animals treated with N-acetylcysteine showed a similar temporary threshold shift but a clear improvement in the recovery of compound action potential thresholds, with significantly reduced permanent threshold shift and hair cell loss. These data suggest that N-acetylcysteine is able to attenuate the toxic effect of acoustic trauma and could represent an interesting molecule for preventing inner ear injuries.

KEY WORDS: Noise-induced hearing loss • Acoustic trauma • Cochlea • Antioxidant • Oxidative stress

RIASSUNTO

Lo stress ossidativo svolge un ruolo rilevante nell'ipoacusia da rumore in quanto partecipa attivamente alla genesi dei meccanismi di morte cellulare che seguono l'esposizione a suoni di elevate intensità e che conducono all'insorgenza di una ipoacusia neurosensoriale. In questo studio sono stati analizzati nel modello animale i meccanismi patologici alla base del danno da rumore ed il ruolo protettivo della molecola ad azione antiossidante N-acetilcisteina. Gli animali sono stati divisi in 2 gruppi, esposti a trauma acustico (6 kHz, 120 dB, 30 minuti) e trattati uno con N-acetilcisteina e l'altro con soluzione salina (gruppo di controllo) per i successivi 3 giorni. La funzione uditiva è stata monitorata mediante registrazioni elettrofisiologiche giornalmente a 2-16 kHz per 7 giorni; l'analisi morfologica è stata eseguita mediante microscopia elettronica a scansione. La soglia uditiva registrata un'ora dopo il trauma acustico negli animali controllo risultava elevata a 70-90 dB per le alte frequenze, con una massima variazione per i 12 e 16 kHz. Durante le prime 24 ore veniva evidenziato un recupero di circa 20 dB fino a raggiungere una elevazione di soglia di 50-70 dB; non venivano registrate ulteriori modificazioni nella settimana seguente. Gli animali trattati con N-acetilcisteina mostravano una variazione di soglia temporanea simile ai controlli, seguita tuttavia da un successivo miglioramento di soglia e riduzione della mortalità delle cellule ciliate esterne. Questi dati suggeriscono un effetto protettivo contro il trauma acustico da parte dell'N-acetilcisteina con un potenziale razionale terapeutico nell'ipoacusia da rumore.

PAROLE CHIAVE: *Ipoacusia da rumore • Trauma acustico • Coclea • Antiossidanti • Stress ossidativi*

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Introduction

Hearing loss due to noise exposure is a common sensorineural hearing impairment in industrialised countries. Hair cell damage induced by intense noise exposure has been widely observed in the organ of Corti, nevertheless the pathways underlying hair cell death following acoustic trauma remain unclear. At present, the mechanisms that lead to noise-induced hearing loss can be classified into two major categories: direct mechanical trauma and metabolic damage to the organ of Corti. High intensity noise of 130 dB sound pressure level causes direct mechanical damage of the sensory hair cells and supporting cells in the organ of Corti [1]. Instead, noise of moderate intensity causes the activation of a complex cascade of biochemical processes in the hair cells that leads to the death of the sensory epi-

thelium. The generation of reactive oxygen species (ROS) is considered to be part of the second mechanism, which also includes ischaemia, excitotoxic damage, metabolic exhaustion and ionic imbalance in the inner ear fluids [2 3]. High levels of free radicals, such as the superoxide anion (O_2^-), the hydroxyl radical (OH), and hydrogen peroxide (H_2O_2), have been found in the cochlea of animals [4] and in industrial workers [5] exposed to different intensities of noise. High levels of ROS activate the up-regulation of cochlear anti-oxidant enzyme activity [6] and modulate the glutathione (GSH) anti-oxidant compound [7 8] (Fig. 1).

The role of antioxidants in protecting the inner ear from environmental damage has been widely studied [9 10] and a variety of agents with antioxidant properties have been shown to attenuate threshold shifts and/or hair cell loss when given prior to, or even shortly after, noise exposure. Research workers have reported certain degrees of protection against noise-induced hearing loss (NIHL) using different antioxidant molecules including glutathione [11]; ebselen [12 13]; alpha-tocopherol [14 15]; D-methionine [16]; acetyl-L-carnitine [17] and many others.

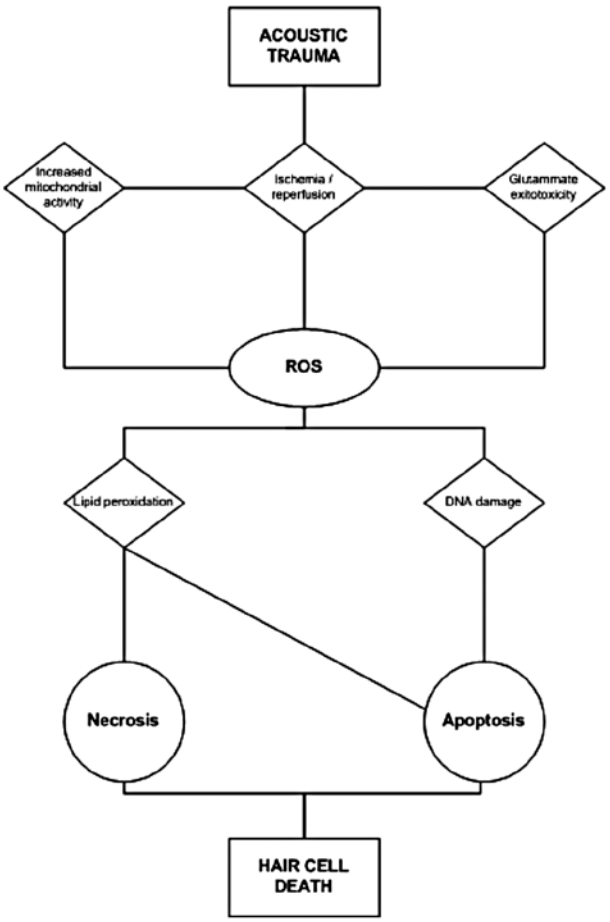

Fig. 1. Molecular pathways underlying hair cell death following acoustic trauma. After noise overexposure, reactive oxygen species (ROS) are generated through increased mitochondrial activity, ischaemia/reperfusion damage and glutamate excitotoxicity. ROS induce lipid peroxidation, followed by rupture of lipid membranes and either hair cell apoptosis or necrosis, and DNA damage, that leads to apoptosis.

N-acetylcysteine (NAC) is a well-known drug used in the clinical setting to treat different diseases such as chronic bronchitis and *keratoconjunctivitis sicca*. Moreover, NAC is a well-tolerated antidote to ROS-induced liver damage due to acetaminophen overdose [18]. NAC has been studied in a variety of *in vitro* and *in vivo* models, addressing most of the currently known mechanisms that may be associated with the genesis of cochlear injury, induced by oxidative stress. In detail, NAC has been documented to act as a free radical scavenger, substrate for GSH production, mitochondrial protectant, glutamate excitotoxicity inhibitor, lipid peroxidation inhibitor, and necrosis inhibitor. Its protective properties have already been documented against acoustic trauma in vivo [19-21] using different protocols of noise exposure and doses of drug, including a recent animal model of high kurtosis noise which simulates workplace noise exposures in manufacturing and industrial facilities [22]. Furthermore, different protocols of NAC injection, before or after noise exposure, have been compared [23 24], demonstrating that post-trauma injection offers a better protection when compared to pre-exposure treatment. Aim of this study was to test the efficacy of NAC in protecting guinea pigs from NIHL when injected immediately and on the 2 days after noise exposure.

Material and Methods

Experimental groups

A total of 18 adult female Hartley albino guinea pigs weighing 250-350 g, with normal Preyer's reflex, were studied. All procedures on animal use and care were conducted in accordance with the Laboratory of Animal Care and Use Committee of the Catholic University of Rome (UCSC), School of Medicine, and were approved by the Italian Department of Health (Ministero della Salute). Animals were divided into two groups; one group (n = 8) was injected intra-peritoneally (i.p.) with saline (control group); the other group (n = 10) was treated with NAC i.p. at a dose of 500 mg/Kg body weight. Body weight of each animal was monitored daily, and the administered dose was adjusted accordingly. Animals were treated immediately after noise exposure and then every 24 h for the following two days. Animals in the control group were injected with a similar volume of saline according to the same schedule as the experimental group.

Noise exposure

Acoustic trauma was induced by a continuous pure tone of 6 kHz generated by a waveform generator (Leader LAG-120 dB, USA) and amplified by an audio amplifier (Pioneer A-307R, Japan). Guinea pigs were anaesthetised (Ketamine 60 mg/Kg body weight, xylazine 2 mg/Kg body weight and acepromazine 0.2 mg/Kg body weight), placed in a soundproof boot and exposed for 30 min to

a 120 dB SPL sound presented binaurally in free field via a loudspeaker (Audax-TW034X0 Tweeter, Chateau du Loir, France) positioned 5 cm in front of the animal's head. Animals' body temperature was maintained within physiological levels by a heating pad, animals were closely monitored during the entire exposure time.

Electrophysiological measurements

Prior to noise exposure and drug administration, anaesthetised guinea pigs (Ketamine 30 mg/Kg body weight; xylazine 1 mg/Kg body weight and acepromazine 0.1 mg/Kg body weight) with normal middle ear were implanted with a round window electrode to record compound action potentials (CAPs) as described elsewhere [25]. To elicit CAPs at 2, 4, 8, 12, 16 and 20 kHz, tone-bursts (1 ms rise/fall time, 10 ms total duration) were delivered at intensities ranging between 0 and 100 dB SPL. A computer-controlled TDT System 3 (Tucker Davis Technologies, Alachua, FL, USA) was used to record CAPs and generate the auditory stimulus. The two groups underwent electrophysiological tests before noise exposure to establish baseline values, 1 hour and every 24 hours thereafter for 1 week after noise trauma. Changes in cochlear function were characterised as CAP threshold shifts.

Morphological studies: scanning electron microscopy (SEM)

Following the final recording session, one week after acoustic overexposure, all animals were sacrificed by means of a lethal injection of anaesthetic and the temporal bone was removed to expose the tympanic bulla. Cochleae were processed for SEM; briefly, cochleae were removed and perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffer, fixed overnight and incubated for 2 h in 2% osmium tetroxide cacodylate buffer (0.1 M). After dissection, cochleae were dehydrated with ethanol, dried with a critical point dryer and coated with gold. Each specimen was viewed and photographed by means of a Zeiss Supra 50 Field Emission apparatus. Hair cells were counted; results were expressed as the percentage of outer hair cells remaining in each row over the entire length of the cochlea.

Statistical analysis

Statistics were performed by means of the analysis of variance (group x frequency x day three-way ANOVA with repeated measures). When significant differences were found with the overall analyses, post-hoc comparisons were assessed using Turkey's test.

Results

Auditory function evaluation

In the control group, the mean hearing thresholds measured 1 hour after acoustic trauma were elevated to 70-90 dB in the higher frequencies of the CAP audiogram, with a maximum threshold elevation ranging between 12 and 20 kHz. During the first 24 h after the acoustic trauma, there was a partial recovery of CAP thresholds of about 20 dB, to reach a final threshold shift elevation of approximately 50-70 dB, but there was no further improvement during the remainder of the experimental week.

When compared to control animals, NAC did not modify the acute effect of the acoustic trauma nor the time course of the functional recovery. In contrast, guinea pigs in the NAC group showed a clear improvement in the recovery of CAP thresholds, with significantly reduced permanent threshold shift. This improvement was evident on the 7th day after the acoustic trauma when the threshold shift in NAC-treated animals was about 20-30 dB; the effect was more pronounced and statistically significant ($p < 0.002$) on middle and high frequencies (Fig. 2).

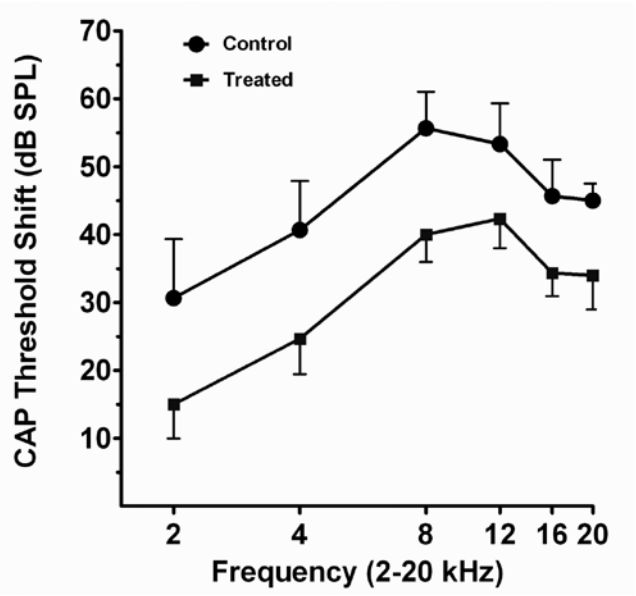

Fig. 2. Mean (± SEM) CAP threshold values in control noise-exposed animals (n = 8) and noise-exposed plus NAC animals (n = 10) 7 days after acoustic overexposure. Average threshold shifts are greater in higher frequencies of CAP audiogram. NAC treatment reduced CAP elevation; this improvement was clearly seen at 7 days following acoustic trauma with statistical significance on mid and high frequencies ($p < 0.002$).

Morphological observations

On the surface preparation of each cochlea, the missing outer hair cells (OHCs) and inner hair cells (IHCs), in the basal, second, third and apical turns, were counted. In the control cochleae (Fig. 3 A-B), a massive loss of hair cells was found in the area located 14-16 mm from the apex, the area corresponding to hair cells coding for 8-14 kHz [26]. Most of the hair cell losses were found in the first OHC row, and to a lesser extent in the second and third OHC rows. Although there was no obvious loss of OHCs, in each turn of each cochlea, disarrayed OHC stereociliary bundles were found on the cochleae exposed to noise. There was no visible loss of IHC stereocilia throughout the entire basilar membrane of each cochlea. Consistently with the physiological data, fewer hair cells were

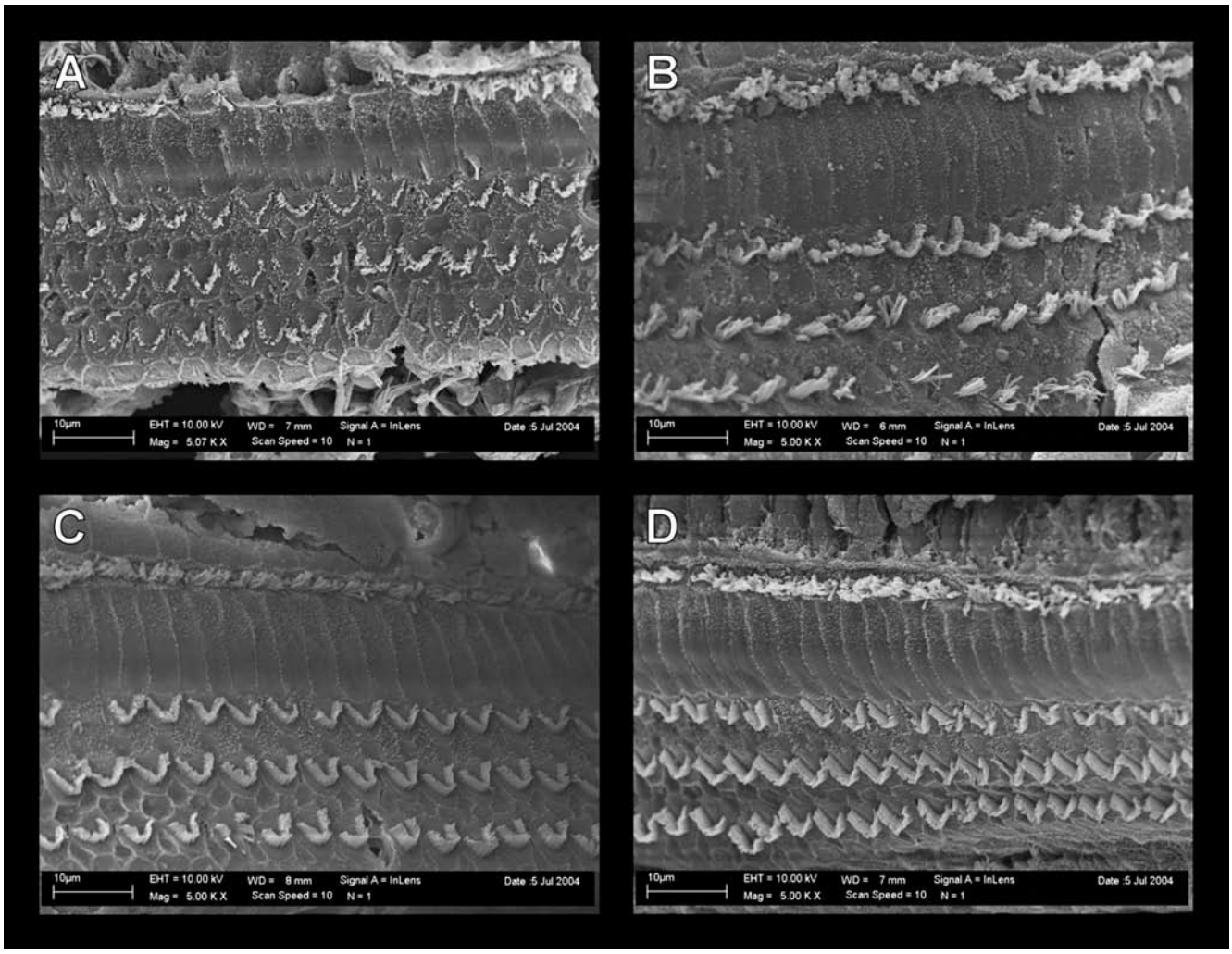

Fig. 3. Surface view of organ of Corti with scanning electron microscopy (SEM) (x 1450). Typical SEM micrographs of areas damaged (A-B) by acoustic trauma from same cochleae. OHCs disappeared and disarrayed OHC stereocilia bundles were observed. In contrast, noise exposed plus NAC showed only a moderate OHC loss in the same regions and stereocilia were normal.

missing when the animals were injected with NAC (Fig. 3 C-D). In detail, 43.4 ± 3.1% of cells were counted in the basal turn of cochleae of control animals, while 80.9 ± 6.8% were counted in animals treated with NAC. In the middle turn, 68.6 ± 5.3% (first portion) and 92.9 ± 4.6% (second portion) of OHC were counted in control animals, while, respectively, 85.8 ± 4% and 94.2 ± 2.6%, were counted in NAC animals. Finally, in the apical turn, 96.5 ± 3% of cells were found in the cochleae of control animals; 98.9 ± 1% were counted in NAC-treated animals (Fig. 4).

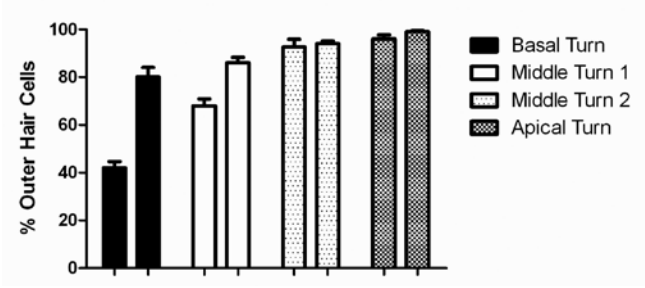

Fig. 4. Hair cell counts of NAC group (n = 20 cochleae) compared to control group (n = 16 cochleae) in basal, middle and apical turns. Results are shown as percentage of intact hair cells for each portion of the cochlea.

Discussion

The present study demonstrates the potential efficacy of NAC in preventing permanent noise-induced threshold shifts after exposing animals to loud continuous sound.

The threshold shift, following noise exposure, reported in this study, was comparable to data in the literature referring to studies in which the same animal model was used [27 28]. Specifically, after intense noise exposure, we found a significant permanent shift that affected, primarily, higher frequencies. In this model of acoustic trauma in the guinea-pig, 30 min of 6 kHz tone at 120 dB SPL induced a substantial hair cell loss; however, maximal hair cell loss was restricted to a precise region located 14-16 mm from the apex, which was the site maximally stimulated.

The use of antioxidants in protecting the inner ear from acoustic trauma has been widely studied. Several Au-

thors observed various degrees of protection provided by glutathione [11], R-phenylisopropyladenosine (R-PIA) [29], superoxide dismutase-polyethlene glycol (SOD) and allopurinol [30]. The results obtained in the present study provide evidence that the administration of NAC resulted in a reduction in threshold shift and hair cell loss following continuous noise, these data being consistent with the results obtained in a model of cisplatin ototoxicity [31 32].

The protection provided by NAC may be directly related to its ability to inhibit lipid peroxidation and scavenge ROS within the cell, indirectly by increasing the intracellular levels of GSH by acting as a cysteine donor to increase GSH synthesis [20], reduce the level of caspases and contrast glutammate excitotoxicity [33 34] (Table I). However, although NAC offers a certain degree of protection, baseline values, prior to the noise-induced CAP threshold shifts, are not fully recovered. This would appear to indicate that besides oxygen-free radicals, some other mechanisms may also contribute to hearing loss following acoustic trauma thus stimulating further experiments in which NAC could be used in conjunction with other agents with possible synergistic protective effects. The present study did not specifically provide data on NAC toxicity or side-effects. Duan et al. [21] observed that a cumulative dose of NAC (1750 mg/Kg), administered before and after impulse noise trauma, resulted in a greater permanent threshold shift and more inner hair cells loss compared to control animals. Instead, a cumulative dose of 1050 mg/Kg, over 5 days, resulted in significant protection against impulse noise. In another experiment, two groups of animals were treated with the two different cumulative doses and were not exposed to acoustic trauma; these animals did not show any threshold shift. Thus, although NAC alone is not toxic for the cochlea, dosage of the drug is critical to elicit its protective effect. In our model, we used a dose of 500 mg/Kg i.p. administered immediately after noise exposure and then during the following two days (cumulative dose of 1500 mg/Kg).

Table I. Potential NAC effects on inner ear pathologic mechanisms following acoustic trauma.

Mechanism	Effect of NAC
ROS generation	ROS scavenger
GSH depletion	Replenishes GSH
Mitochondrial injury	Protects mitochondria
Caspase activation	Reduces caspases
Glutammate exitotoxicity	Reduces toxicity

Systemic delivery of an otoprotective molecule can compromise the effectiveness by producing undesired side-effects. Intra-cochlear, or via round window transport, administration of the compound may avoid undesired side-effects possibly resulting from systemic application. However, NAC has already been used clinically by means of local administration and/or systemically in large oral doses with no significant side-effects; in addition, the molecular weight of NAC is low enough to allow it to be transported across the round window membrane.

In terms of protection against NIHL, besides the dose, the anti-oxidant timing and mode of administration are also critical. Kopke et al. [19] and Ohinata et al. [20] demonstrated the efficacy of NAC alone. or in association with another antioxidant, if administered before the acoustic trauma. Duan et al. [21] also observed protective effects treating animals before and after the acoustic trauma. Coleman et al. [17] investigated the protective effect of NAC in noise-exposed chinchillas, administering the drug 1, 4 or 12 hours after trauma and reported a certain protection in animals treated with NAC only when the drug was given 1 hour after noise exposure, while no protection was observed in animals treated 4 or 12 hours after acoustic trauma. In a different study, Hamernik et al. [1] evaluated the difference in auditory evoked potential recordings from the inferior *colliculus* (IC) in animals treated with NAC versus controls; no statistical difference could he highlighted at the IC level. One recent study, in humans, by Kramer et al. [35] reported no significant differences in distortion product otoacustic emissions (DPOAEs) measured in subjects exposed to loud music; participants were given either 900 mg of NAC or placebo 30 minutes before exposure. More recently, Lorito et al. [24] also reported that the administration of NAC, in a NIHL animal model, significantly reduced the threshold shifts in the treated animals, and that the role played by the timing of NAC injection was important for OHC protection; measuring DPOAEs in treated and control animals revealed that the best protection scheme was observed in the group receiving NAC after noise exposure. Our results provide evidence that NAC is effective if administered immediately after noise exposure and in the following days.

In conclusion, NAC is a safe and effective molecule which provides protective effects against acoustic trauma in our NIHL animal model. Nevertheless, further information concerning the most beneficial methods of administration, the dose of NAC and an eventual association with other compounds are essential in order to offer adequate protection against all components of noise trauma.

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