INTRODUCTION

Hearing loss is the most common form of sensory impairment in humans, affecting 360 million persons worldwide, with a prevalence of 183 million adult males and 145 million adult females. Although approximately 1 in 500 children is born with impaired hearing, sudden or progressive forms of hearing loss can manifest at any age. Hearing loss can be caused by environmental factors, such as exposure to noise or ototoxic chemicals, or by aged related senescence.

Traumatic injury, such as injury caused by exposure to an explosion or to the firing of a gun, can lead to sudden hearing loss. Sometimes this hearing loss is accompanied by the perception of a constant ringing noise called tinnitus. Moreover, genetic factors as mutations in MT-TS1, MYO7A, or ACTG1 genes, between many others, have already been linked to nonsyndromic hearing loss. Noise exposure is responsible for approximately 10% of hearing loss in adults, in particular military veterans. Short impulses of high-intensity noise such as a gunshot or explosion can trigger sudden hearing loss, which is generally irreversible and associated with structural damage to the auditory system. Moreover, gene association studies using candidate-gene approaches have focused mostly on genes that are linked to oxidative stress, K+ recycling, and the heat shock response.

On the other hand, various chemical agents as aminoglycoside antibiotics, platinum-containing chemotherapy agents, and nonsteroidal anti-inflammatory drugs as aspirin are ototoxic. For example, aminoglycosides, antibiotic with broad-spectrum activity, cause significant hearing loss, with estimates of a 20–50% chance of incidence when treating acute infections. Hair cells are readily damaged by this compound, probably due to the similarity of hair cell mitochondrial ribosomes to their bacterial counterparts. Furthermore, compounds as cisplatin and carboplatin are extremely ototoxic, particularly in children. Hair cells seem to be highly sensitive to this chemotherapy-induced apoptosis.

Finally, the most common form of sensory impairment in older people is age-related hearing loss. Although hearing loss...
has been considered to be part of a natural ageing process, not all humans suffer from age-related hearing loss; heritability studies suggest that the source of variability is both genetic and environmental. Interestingly, statistical studies have established associations between age-related hearing loss and genes linked to reactive oxygen species (ROS) detoxification,[16] strongly confirming an essential role of mitochondrial oxidative stress in hearing loss.

High oxidative stress has been universally observed in noise-induced cochlear and auditory nerve damage and noise-induced acoustic trauma is also directly associated to increase of ROS in rodents, mimicking human hearing disorders. For this reason, we hypothesized that N-acetylcysteine (NAC), a potent antioxidant able to scavenge a big number of oxygen species,[17] could be an effective therapeutic agent to block the activation of noise-induced hearing loss (NIHL) mechanisms.

In guinea pig, acoustic trauma also induces permanent hearing loss characterized by a permanent auditory brainstem response (ABR) threshold shift and decrease of distortion product otoacoustic emission (DPOAE) amplitude, making this guinea pig protocol a robust and reproducible model to study the efficacy of new drug candidates in hearing impairment induced by noise exposure. In this study, we reproduced Fetoni et al., 2009; acoustic trauma protocol and we confirmed the presence of permanent hearing impairment and an increase of prestin biomarker 15 days after acoustic trauma in guinea pig. Moreover, we observed that NAC treatment partially reduces the hearing loss induced by acoustic trauma when administrated daily at 500 mg/kg by oral gavage.

MATERIALS AND METHODS

Animal housing and drug administration
Male Hartley albino guinea pig (Envigo, France) was kept in the A1 animal house facility. Animals were housed in ventilated and clear plastic boxes and subjected to standard light cycles (12 h in 90-lux light, 12 h in the dark). NAC (Sigma Aldrich, A7250-50G) was diluted in water at 0.1 g/mL. The animals were treated by oral gavage at 500 mg/kg (Sigma Aldrich, A7250-50G) was diluted in water at 0.1 g/mL. The animals were treated by oral gavage at 500 mg/kg every day during 15 days [Figure 1]. All animal experiments were approved by the CEEALR, Montpellier, France.

Acoustic trauma
Acoustic trauma was induced individually by a continuous sound at 6 kHz at 120 dB for 1 h. The stimuli were delivered bilaterally in anesthetized animals using earphones previously calibrated in an acoustic box allowing homogeneous sound administration. We reproduced the experimental acoustic trauma conditions published by Fetoni et al. in 2009.[18,19]

ABR
For ABR studies, guinea pigs were anesthetized using ketamine/xylazine mixture, and body temperature is regulated using a heating pad at 37°C. Then, earphones were placed in the left ear of each animal, an active electrode was placed in the vertex of the skull, a reference electrode under the skin of the mastoid bone, and a ground electrode was placed in the neck skin. The stimuli consisted of tone pips of six frequencies (2 kHz, 4 kHz, 6 kHz, 12 kHz, 16 kHz, and 24 kHz) at various sound levels (from 0 to 90 dB). ABR measures of each animal were performed individually and using OtoPhyLab system.

DPOAE
Guinea pig was anesthetized using ketamine/xylazine mixture and an probe (OtoPhyLab) was inserted into the external left ear canal. The primary tone F2 was set at six frequencies (2 kHz, 4 kHz, 6 kHz, 12 kHz, 16 kHz, and 24 kHz). The frequency ratio F2/F1 was set at 1.2. At all frequencies (F2), the input of DPOAE systems were received, digitized, and evaluated using the output of the microphone.

Enzyme-linked immunosorbtent assay (ELISA) prestin quantification
For each animal, 50 µl of blood was sampled by saphenous vein puncture and collected in a tube containing ethylenediaminetetraacetic acid as anticoagulant. Samples were centrifuge and the supernatant (plasma) was diluted at 1/10 and prestin quantification for each animal was performed by ELISA method.

Western blot
Plasma samples were denaturalized and separated in 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membrane. Membrane was blocked for 60 min with LI-COR Blocking Buffer. The membrane was incubated overnight with rabbit anti-β-tubulin (1:100) and mouse anti-prestin (1:100). The following day the membrane was washed in TBS Tween-20 (0.1% V/V) and incubated with the secondary fluorescence antibodies: Donkey anti-rabbit IRDye 800 (1:10.000) and Goat anti-mouse IRDye 680 (1:10,000). Then, the membrane was washed in TBS Tween-20 (0.1% V/V) and visualization of the bands was performed using LI-COR scanning device.

Statistics analysis
Data are represented as mean ± SEM. Statistical significance was determined using 2-way ANOVA, followed by a Dunnett’s multiple comparison post-hoc test. P < 0.05 was considered statistically significant. n = 8 animals/group at the baseline and n = 7 animals/group after acoustic trauma.
RESULTS

NAC treatment reduced the permanent increase of ABR thresholds

Hearing performances were analyzed at day 1 (baseline) and 24 h and 15 days after acoustic trauma. The acoustic trauma+NAC group was treated with NAC solution at 500 mg/kg once a day during 15 days [Figure 1].

Similar ABR thresholds were observed in all groups at the baseline [day 1] [Figure 2a]. The ABR thresholds of all animals were <40 dB at 6 kHz, so no animals have been excluded at the baseline, considering that all animals had good hearing capacities.

At day 2 (24 h after acoustic trauma), the ABR thresholds of both acoustic trauma groups were significantly increased at 6 kHz, 12 kHz, 16 kHz, and 24 kHz compared to the control group. Since the guinea pig numbers 10 and 21 presented an ABR threshold shift <20 dB at 6 kHz, these animals were considered not sensitive to acoustic trauma and were excluded from the study. As expected, we observed around 12% of animal exclusion. At this time point, each acoustic trauma group was composed by 7 guinea pigs. At the analyzed frequencies, no significant differences were observed between the vehicle and NAC group at day 2 except at 12 kHz [Figure 2b].

At day 15, the ABR thresholds of the acoustic trauma+vehicle group were significantly increased at 4 kHz, 6 kHz, 12 kHz, and 16 kHz compared to the control group showing the presence of a permanent hearing impairment 15 days after acoustic trauma [Figure 2c]. The acoustic trauma+NAC group also presented a significant increase of ABR threshold at 4 Hz, 6 kHz, 12 kHz, and 16 kHz compared to the control group. However, the ABR thresholds of NAC treated group were significantly lower that the vehicle-treated group at 6 kHz, 12 kHz, and 16 kHz suggesting a partial protective effect of NAC in hearing impairment induced by acoustic trauma in guinea pig.

NAC treatment increased DPOAE amplitude after acoustic trauma

The functionality of the outer hair cells was determined using DPOAE. Similar DPOAE amplitudes were observed in control and acoustic trauma groups at the baseline (day 1) [Figure 3a].

At day 2 (24 h after acoustic trauma), the DPOAE amplitudes of the acoustic trauma+vehicle group were significantly decreased at 6 kHz, 12 kHz, and 16 kHz compared to the control group. Moreover, the acoustic trauma+NAC group also presented a significant decrease of DPOAE amplitude at 6 kHz and 12 kHz compared to the control group. No significant differences between vehicle and NAC treated animals were observed at the analyzed frequencies 24 h after acoustic trauma [Figure 3b].

Finally, at day 15, the DPOAE amplitude of the acoustic trauma+vehicle group remained significantly decreased at 6 kHz, 12 kHz, and 16 kHz compared to the control group.
demonstrating a lack of functions of the cochlear outer hair cells and confirming the presence of a permanent hearing impairment 15 days after acoustic trauma [Figure 3c]. Moreover, even if no statistical differences were observed between NAC and vehicle-treated groups at the analyzed frequencies probably due to the biological variability, the decrease of DPOAE amplitude of NAC treated group was not statistically significant compared to the control group [Figure 3c], corroborating ABR data and suggesting a partial protective effect of NAC treatment in hearing impairment induced by noise.

Acoustic trauma increased plasma prestin biomarker

Prestin has been described as a new hearing loss plasma biomarker.[20] For this reason, we studied plasma prestin in guinea pig before and after acoustic trauma. Time-lapse western blot analysis of prestin demonstrated an increase of the prestin biomarker after acoustic trauma. Even if no statistical differences were observed between vehicle and NAC treated group 24 hours after acoustic trauma (day 2), the concentration of prestin of NAC treated animals was significantly lower compared to the vehicle-treated group at day 15 [Figure 4a] confirming a protective effect of NAC treatment.
ELISA analysis of plasma also demonstrated a significant increase of the prestin biomarker at 323.2 ± 15.0 pg/mL of plasma in the acoustic trauma+vehicle group, whereas the prestin concentration in control animals was 123.2 ± 14.2 pg/mL of plasma at day 15 [Figure 4b]. Moreover, the NAC treated group also presented a significant increase of plasmatic prestin at 262.9 ± 11.9 pg/mL compared to the control group, but this increase was significantly lower than vehicle-treated animals [Figure 4b], confirming that NAC treatment reduces prestin hearing loss biomarker in guinea pig 15 days after acoustic trauma.

**DISCUSSION**

Cochleae are extremely vulnerable to oxidative stress because of the high metabolic demands of their mechanosensory hair cells in response to sound stimulation. Normally, ROS produced by hair cell mitochondria during physiological conditions is scavenged by hair cell endogenous antioxidant mechanisms. However, in noise conditions, the expression of ROS species increases, leading to the increase of oxidation and genetic and cellular alterations which cause cellular dysfunctions and progressive cochlear degeneration.[21,22]

In this way, antioxidants such as glutathione, d-methionine, resveratrol, and ascorbic acid all attenuated NIHL in animal models when given before noise exposure.[23] Interestingly, polymorphisms in the gene encoding catalase have been linked to an increased susceptibility to hearing loss in humans, and mice that are heterozygous for a mutation in Sod1 gene show an increased vulnerability to hearing loss induced by noise exposure.[24] These genetic findings provide additional evidence that antioxidant treatment might be crucial for the maintenance and recovery of the normal hearing under loud noise conditions. Taken together, these data suggest that the therapy focusing in the reduction of this increase of oxidative stress in cochlea could be feasible options for the treatment of several types of hearing loss.

Corroborating this hypothesis, some clinical and military trials have been carried out for a temporary threshold shift, in which administration of antioxidant, nutritional supplements before moderate noise exposure showed some beneficial effects.[25] However, clinical trials using NAC remain presently controversial and inconclusive. Whereas Kramer and collaborators published that NAC treatment did not provide protection from temporary thresholds shifts after noise exposure,[26] Kopke et al. demonstrated that NAC significantly reduced auditory threshold shifts and DPOAE changes in military subjects undergoing routine weapons training.[27] However, whereas our results suggest a preventive effect of NAC in the NIHL process until now, no long-term preventive clinical studies have been carried out in humans. For this reason, the development of new antioxidant compounds is essential to understand the role of oxidative stress in hearing impairment and to prevent NIHL in the future.

**CONCLUSION**

In this study, we observed a permanent hearing impairment and increase of plasma prestin biomarker in guinea pig 15 days after acoustic trauma. Corroborating previous publications, we demonstrated that NAC treatment partially reduced the hearing loss induced by acoustic trauma when administrated daily at 500 mg/kg by oral gavage. Our data reproduced Fetoni et al. 2009 publications[18,19] confirming that acoustic trauma at 6 kHz at 120 dB during 1 h leads to a permanent hearing loss in guinea pig. This is a robust and reproducible model allowing to determine the efficacy of new pharmacological candidates targeting noise-induced hearing impairment in guinea pig.

**REFERENCES**

12. Agrawal RK, Sharma MR. Structural aspects of mitochondrial...

How to cite this article: Gonzalez-Gonzalez S, Cazevieille C. N-acetylcysteine Treatment Reduces Noise-induced Hearing Loss in Guinea Pig. J Community Prev Med 2020;3(1):1-6.