Am J Transl Res 2024;16(1):272-284
www.ajtr.org /ISSN:1943-8141/AJTR0154052

Original Article

ROS-induced oxidative stress and mitochondrial dysfunction: a possible mechanism responsible for noise-induced ribbon synaptic damage

Zi-Jing Yang[1,2], Chun-Li Zhao[1,2], Wen-Qi Liang[1,2], Zhong-Rui Chen[1,2], Zheng-De Du[1,2*], Shu-Sheng Gong[1,2*]

[1]Department of Otolaryngology Head and Neck Surgery, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China; [2]Clinical Center for Hearing Loss, Capital Medical University, Beijing 100050, China. [*]Equal contributors.

Received October 23, 2023; Accepted January 5, 2024; Epub January 15, 2024; Published January 30, 2024

Abstract: Evidence suggests that damage to the ribbon synapses (RS) may be the main cause of auditory dysfunction in noise-induced hearing loss (NIHL). Oxidative stress is implicated in the pathophysiology of synaptic damage. However, the relationship between oxidative stress and RS damage in NIHL remains unclear. To investigate the hypothesis that noise-induced oxidative stress is a key factor in synaptic damage within the inner ear, we conducted a study using mice subjected to single or repeated noise exposure (NE). We assessed auditory function using auditory brainstem response (ABR) test and examined cochlear morphology by immunofluorescence staining. The results showed that mice that experienced a single NE exhibited a threshold shift and recovered within two weeks. The ABR wave I latencies were prolonged, and the amplitudes decreased, suggesting RS dysfunction. These changes were also demonstrated by the loss of RS as evidenced by immunofluorescence staining. However, we observed threshold shifts that did not return to baseline levels following secondary NE. Additionally, ABR wave I latencies and amplitudes exhibited notable changes. Immunofluorescence staining indicated not only severe damage to RS but also loss of outer hair cells. We also noted decreased T-AOC, ATP, and mitochondrial membrane potential levels, alongside increased hydrogen peroxide concentrations post-NE. Furthermore, the expression levels of 4-HNE and 8-OHdG in the cochlea were notably elevated. Collectively, our findings suggest that the production of reactive oxygen species leads to oxidative damage in the cochlea. This mitochondrial dysfunction consequently contributes to the loss of RS, precipitating an early onset of NIHL.

Keywords: Noise-induced hearing loss, mitochondrial dysfunction, reactive oxygen species, oxidative stress, ribbon synapses

Introduction

Recent reports suggest that approximately one-fifth of the global population experiences varying degrees of hearing loss, making deafness the third-most prevalent disabling health condition [1, 2]. Noise exposure (NE) is one of the main causes of hearing loss. Earlier studies generally accepted that hearing loss is directly caused by high-intensity noise stimulation, which damages sensory hair cells (HCs) [3, 4]. However, recent studies have found that moderate NE can also lead to a temporary hearing threshold shift (TTS) and a decline in speech recognition rates, which affects the patients' quality of life [5]. Due to individual variability and the complexity of noise, the underlying mechanisms of noise-induced hearing loss (NIHL) warrent further exploration.

Hearing production relies on the vibration of the cochlear membrane and the associated potential changes. As the principal acoustic receptor, the cochlea's functionality is crucial for hearing formation. NE impacts the cochlea through two primary mechanisms, as follows. 1) Mechanical Damage: Sound vibration drives the tectorial membrane and HCs to oscillate. However, prolonged high-intensity noise can disrupt this arrangement, leading to the detachment of the organ of Corti and irreversible damage to the inner ear's structure [6, 7]. 2) Metabolic

https://doi.org/10.62347/EVDE9449

Impairment: In eukaryotes, mitochondria are central to aerobic respiration, consuming oxygen to produce ATP. Despite this process's efficiency, it invariably generates reactive oxygen species (ROS) such as peroxides, superoxide, and hydroxyl radicals that are produced continuously along the electron transport chain. These ROS can induce oxidative stress and mitochondrial dysfunction [8, 9]. Studies have shown that ROS-induced DNA damage accelerates cochlear cellular senescence and contributes to age-related hearing loss (ARHL) [10]. Additionally, ototoxic drug use may exacerbate ROS production, leading to HC death [11]. Furthermore, HCs in the cochlea of animals lacking antioxidant enzymes are more susceptible to NE [12]. All the examples above suggest that the accumulation of ROS is associated with the development of sensorineural hearing loss.

Conversely, recent insights challenge the long-held belief that HCs are the most vulnerable structures in the cochlea. It is now understood that synaptic connections between inner hair cells (IHCs) and cochlear nerve fibers are more susceptible to early disruption before hearing loss onset [13, 14]. NE triggers excessive glutamate release in the synaptic cleft, causing glutamate excitotoxicity. The correlation between synapse numbers and mitochondrial density highlights the critical role of mitochondria in maintaining ribbon synapses (RS) functionality. Evidence suggests cochlear RS damage as an early contributor to ARHL [15, 16]. Additionally, prolonged or frequent exposure to moderate noise also damages the RS [17, 18]. While there is no direct evidence linking ROS to the loss of RS, studies show a significant decrease in cochlear mitochondrial membrane potential (MMP) with increased ROS production in hydrogen peroxide (H_2O_2)-treated cochlear samples, accompanied by RS loss and HC apoptosis [19]. Antioxidants have also been used to prevent damage in various models of hearing loss [20, 21]. Therefore, it is reasonable to presume that ROS-induced oxidative mitochondrial damage is associated with the loss of RS, which also aids in the development of NIHL.

Considering the important role of mitochondrial dysfunction in NIHL development, we aimed to investigate whether noise-induced oxidative stress is a key factor in synaptic damage within the inner ear. To prove this conjecture, we performed mice to single or repeated NE and assayed oxidative stress-related indicators, thus further investigating the relationship between ROS and NIHL.

Methods

Animals and NE

Six-week-old male C57BL/6J mice were obtained from the Experimental Animal Center of Capital Medical University (Beijing, China). All the mice were housed and bred in a 12 h dark and quiet environment, with a maximum of 5 mice per cage, and were allowed to eat and drink freely. After excluding abnormal mice in the primary hearing test, mice were placed in the middle of two opposite speakers in a soundproof chamber and exposed to 100 dB sound pressure level (SPL) broadband white noise for 2 h. The XTi4002, CROWN amplifiers (Harman, Elkhart, IN) were connected to the mixer and the sound was edited and synthesized by Cool Edit Pro software (Adobe Systems, San Jose, CA). Two weeks after that, half of those mice went through another NE with the same condition (**Figure 1A**). Age-matched control group mice were kept in silence during this study and all the protocols were approved by the Committee on the Ethics of Animal Experiments of Capital Medical University.

Auditory brainstem response (ABR) test

The auditory function of mice was evaluated on the following three time points: post-exposure day 1, post-exposure day 14, and 2 weeks after the second NE. Before testing, mice were anesthetized by intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture and were placed in a soundproof chamber. A reference electrode was inserted into the mastoid of the ear to be tested, the recording electrode was inserted under the skin in the middle of the cranial top, and the ground electrode was inserted behind the mastoid of the opposite ear. ABR responses were measured with click and a tone burst stimulus at frequencies of 4, 8, 16, and 32 kHz with the TDT System 3 (Tucker-Davis Technologies, Alachua, FL, USA). Acoustic stimuli were initially presented at 90 dB SPL and decremented in 5 dB SPL steps to approximately 5 dB SPL below the threshold. The threshold was defined as the

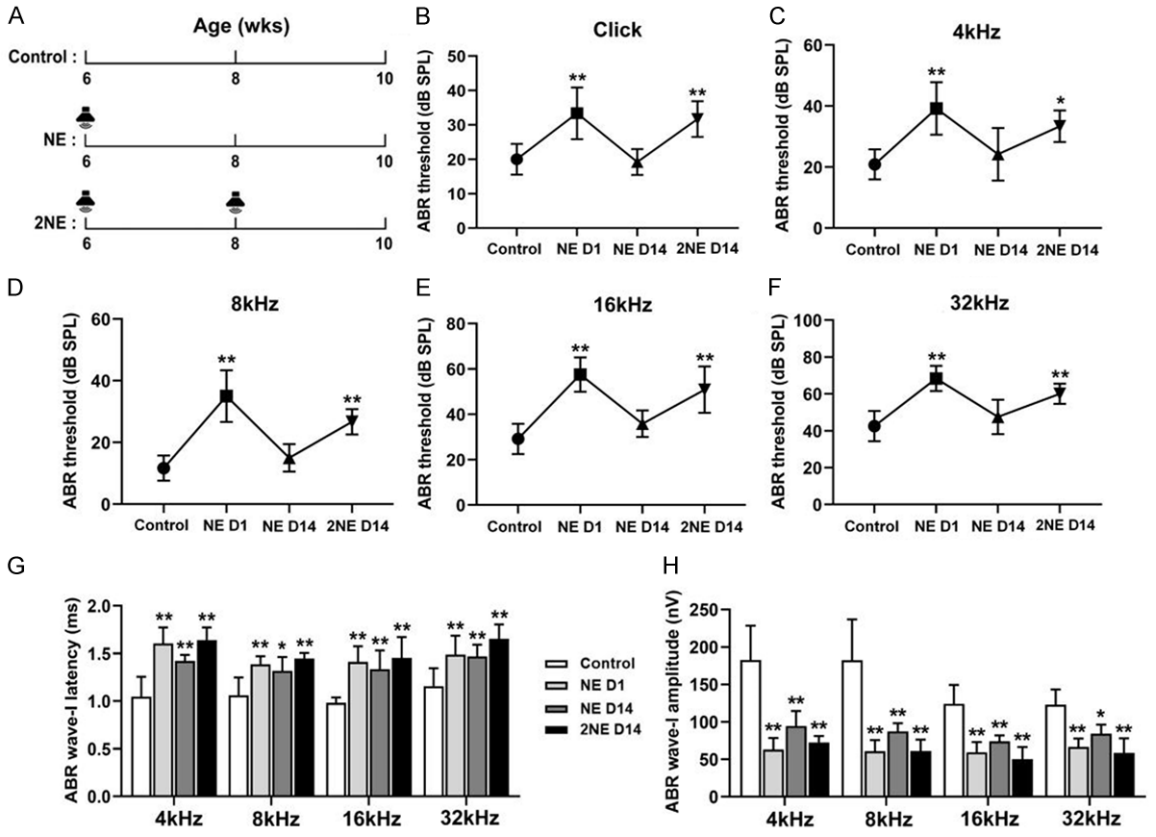

Figure 1. Noise exposure (NE) causes a negative effect on auditory function in mice. A. NE protocol. During the course of the experiment, mice were given frequent exposure to 100 dB SPL white noise for 2 hours at each point marked in the image. ABR tests were performed within 24 hours or 2 weeks after each NE. B-F. ABR thresholds change at Click, 4 k, 8 k, 16 k, and 32 kHz frequencies. Compared with the control group, mice showed a significant hearing threshold shift after NE at all frequencies, with hearing recovery after two weeks. However, after a second NE, the hearing threshold could not return to normal levels. G. Analysis of ABR wave I latencies changes after NE at each frequency. Compared with the control group, the wave I lantencies were significantly prolonged at each frequency within 24 h or 2 weeks after single or repeated NE. H. Analysis of ABR wave I amplitudes changes after NE at each frequency. Compared with the control group, a significant decrease in wave I amplitudes were shown at each frequency within 24 h or 2 weeks after single or repeated NE. *P < 0.05; **P < 0.01 versus the control group. NE, noise exposure; ABR, auditory brainstem response.

lowest intensity of stimulation that yielded a repeatable waveform based on an identifiable ABR wave. The latencies and amplitudes of wave I at 90 dB SPL were recorded. All of the above tests and hearing assessments were done by the same person.

Cochlear tissue preparation

For basement membrane pavement staining, mice were sacrificed by spinal dislocation under anesthesia. Cochlear tissue was rapidly removed into the 4% paraformaldehyde at 4°C for overnight fixation after removing excessive tissue under a microscope. After decalcification in 10% ethylenediaminetetraacetic acid (EDTA) for 2 h, structures such as the cochlea shells and capping membranes were carefully removed under the microscope. The remaining basilar membranes were divided into three turns (apical, middle, and basal) for the next staining.

For frozen section staining, cochleae were decalcified with 10% EDTA for 48 h after being fixed overnight with 4% paraformaldehyde at 4°C. The prepared samples were then dehydrated in a 30% sucrose solution at 4°C for 30 minutes and embedded in an optimal cutting temperature compound. Finally, the samples were sectioned at a thickness of 10 μm using a Leica Cryostat (Germany).

Immunostaining protocols

Prepared samples were incubated with 5% normal goat serum (ZSGB-BIO, Beijing, China) and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline for 2 h at room temperature. The corresponding primary and secondary antibodies were subsequently incubated.

For basement membrane pavement staining, the primary antibodies were as follows: rabbit anti-Myosin VIIa (diluted 1:300; Proteus Biosciences, NJ, USA); mouse anti-C-terminal binding protein 2 (CtBP2, diluted 1:300; BD Biosciences, NJ, USA). After washing with PBS three times on the following day, samples were incubated with goat anti-rabbit IgG (H+L) Alexa Fluor 647 (diluted 1:300, Invitrogen, CA, USA) and goat anti-mouse IgG1 Alexa Fluor 568 (diluted 1:300, Invitrogen, CA, USA) secondary antibodies for 2 h in darkness.

For frozen section staining, the primary antibodies were as follows: mouse anti-4 hydroxynonenal (4-HNE, diluted 1:200; Abcam, Cambridge, UK); mouse anti-8-hydroxy-2'-deoxyguanosine (8-OHdG, diluted 1:300; Abcam, Cambridge, UK); mouse anti-β-Tubulin III IgG2a (diluted 1:300; Abcam, Cambridge, UK); rabbit anti-Myosin VIIa (diluted 1:300; Proteus Biosciences, NJ, USA). The secondary antibodies used were goat anti-mouse IgG1 Alexa Fluor 568 (diluted 1:300, Invitrogen, CA, USA) and goat anti-rabbit IgG Alexa Fluor 647 (diluted 1:300, Invitrogen, CA, USA).

All the samples were added a drop of 4, 6-diamidino-2-phe-nylindole (DAPI) before being observed under a Leica scanning laser confocal microscope. The fluorescence intensity of 8-OHdG and 4-HNE were measured with the Image J software.

HCs, spiral ganglion cells (SGCs), and synapses counting

The basilar membranes were divided into three turns (apical, middle, and basal). The numbers of OHCs, IHCs, and RS in each turn were counted separately and compared with the control group. We analyzed the number and proportion of lost HCs under the ×63 oil immersion objective lens. Images were scanned at 0.5 μm/layer intervals from the top to the bottom of the IHCs. Myosin VIIa (grey) was used to identify IHCs and CtBP2 (red) was used to label RS. The total number of synapses in each turn was counted for a total of about 10 IHCs, and the average was then determined. We also used frozen section samples to calculate the number of SGCs. Images were scanned at 1 μm/layer intervals by laser confocal microscope. β-Tubulin III (green) was used to identify SGCs and every SGC in the visual field was counted.

H_2O_2, total antioxidant capacity (T-AOC), and ATP assay

Cochlear tissue samples were added to the appropriate reagents according to the manufacturer and centrifuged at 12,000 ×g for 5 min at 4°C. Supernatant was taken for subsequent determination. Protein concentrations were determined by Pierce™ BCA Protein Assay Kit (Thermo, MA, USA). H_2O_2 and T-AOC were then assayed by a Hydrogen Peroxide Assay Kit (Beyotime, Shanghai, China) and a T-AOC Assay Kit (Beyotime, Shanghai, China), respectively.

ATP concentrations were detected with an enhanced ATP assay kit (Beyotime, Shanghai, China). According to the manufacturer's recommendations, the relative luminescence of each sample was measured with a luminometer. The concentration of ATP was calculated according to an ATP standard curve and expressed as nmol/mg protein.

MMP levels measurement

To evaluate mitochondrial function, MMP levels were measured with a JC-1 assay kit (Beyotime, Shanghai, China). The cochlea of each mouse was rapidly removed and ground with the addition of tryptic digestive enzymes, and the cell suspension prepared by centrifugation was used for subsequent experiments. Suspended cells were resuspended in a cell culture medium containing JC-1 staining solution and incubated at 37°C for 20 minutes according to the manufacturer, and then analyzed by flow cytometry (FACS Aria IIu, BD Biosciences).

Statistical analysis

Data are presented as the mean ± standard deviation. A two-way analysis was used to test for more than three groups and multiple com-

parisons were evaluated with the one-way analysis of variance (ANOVA). GraphPad Prism software (version 9.0) was used to plot the experimental results. Differences were considered statistically significant at $P < 0.05$.

Results

Noise-induced auditory dysfunction in mice

To investigate the impact of NE on hearing in mice, we used the ABR test to assess the hearing thresholds and wave I changes across various frequencies. Our findings indicated a significant increase in thresholds after the first day of NE, which returned to normal by post-exposure day 14, suggesting that mice experienced a TTS following the initial NE. However, thresholds did not return to baseline following a second NE under identical conditions, indicating a permanent threshold shift (PTS) (**Figure 1B-F**). Moreover, we evaluated the latencies and amplitudes of the ABR wave I, which represents synaptic function [22]. Notably, unlike the hearing threshold shifts, changes in ABR wave I latencies and amplitudes did not revert to normal two weeks post first NE. There was a marked prolongation in wave I latencies and a decrease in amplitudes. Similar trends persisted following the second NE, suggesting the development of synaptic dysfunction (**Figure 1G**, **1H**). Altogether, these results demonstrat that even moderate NE could negatively affect the auditory function in mice, particularly with repetitive exposure. Notably, synaptic dysfunction occurs earlier in mice than changes in hearing thresholds.

Noise-induced ribbon synapses damage in the cochlea

To discern morphologic changes in the cochlea linked to noise-induced hearing compromise, we collected cochlear samples from mice on post-exposure days 1 and 14, and again 14 days following a second NE, for immunofluorescence staining. After counting the number of HCs, SGCs and RS, we found that neither single nor repeated NE had an effect on SGCs and IHCs (**Figures 2B**, **2D** and **3B**). There was no significant loss of OHCs observed on days 1 and 14 following the initial NE. However, a notable loss of OHCs located in the basal turns was observed 14 days after the second NE (**Figure 2A**, **2C**). Furthermore, compared to the control group, the RS number on post-exposure day 14 notably decreased in the middle and basal turns of the cochlea, especially in the mice that experienced secondary NE. This reduction was significant across all cochlear turns, indicating exacerbated damage from NE (**Figure 3A**, **3C**). In alignment with the ABR test results, these observations confirm that NE indeed causes early damage to cochlear RS, preceding detectable damage to OHCs, IHCs, and SGCs. Furthermore, repeated NE exerted a more pronounced effect on inner ear cell integrity.

Noise-induced mitochondrial dysfunction in the cochlea

To elucidate the mechanisms underlying noise-induced synaptic damage, we further investigated mitochondrial function. We first measured the levels of T-AOC and H_2O_2 to determine the extent of intracellular oxidative stress in the cochlea. The results showed that compared with those in the control group, T-AOC levels did not change significantly on the day following NE. However, the levels markedly decreased by post-exposure day 14 and remained at low 14 days after the second NE, suggesting a decrease in the antioxidant capacity (**Figure 4A**). Additionally, we observed a rapid increase in the H_2O_2 levels on the day following the first NE. Although there was a tendency to recover on post-exposure day 14, levels remained higher than normal and further increased after the second NE (**Figure 4B**). To confirm mitochondrial dysfunction, we further measured the MMP and ATP levels. Post-NE, especially after repeated exposures, mice exhibited a lower percentage of JC-1 aggregates and a higher percentage of JC-1 monomers, indicating reduced MMP (**Figure 4C-E**). Similarly, the ATP levels were notably lower by post-exposure day 14, which were expressed more pronounced two weeks after the second NE (**Figure 4F**).

Taken together, our results indicate that NE leads to a reduced antioxidant capacity in cochlear cells, resulting in the accumulation of H_2O_2. This points to noise-induced mitochondrial dysfunction as a likely contributor to this phenomenon.

Noise-induced oxidative stress in the cochlea

To assess oxidative stress in the cochlea, we analyzed the ROS marker 4-HNE and DNA oxidative damage marker 8-OHdG [23]. Frozen

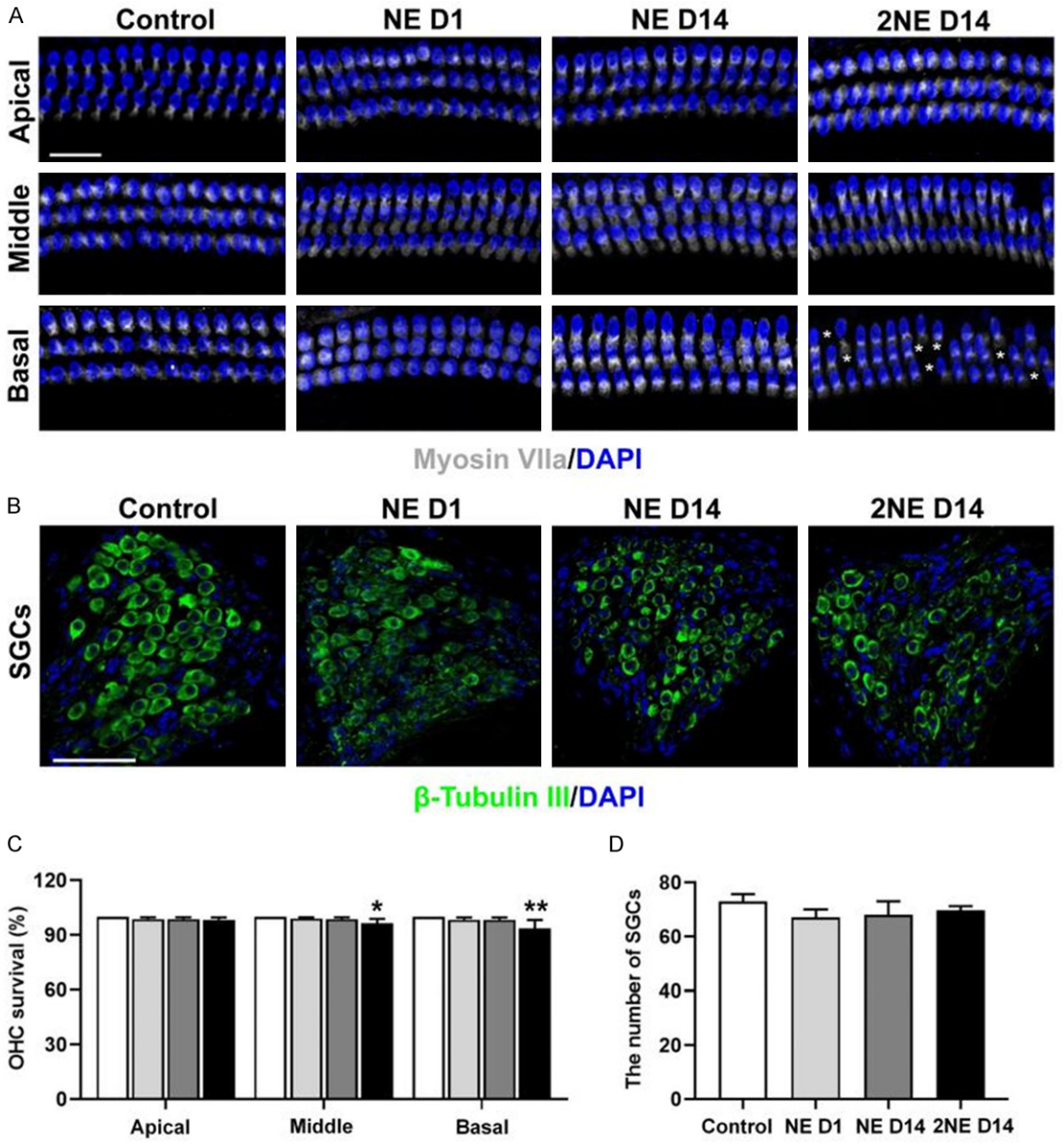

Figure 2. Repeated noise exposure (NE) exacerbates outer hair cells (OHCs) damage but has no effect on spiral ganglion cells (SGCs). A. Representative image of immunofluorescence staining of OHCs after second NE. Scale bar = 20 μm. B. Representative image of SGCs after NE in different groups. Scale bar = 40 μm. C. Survival percentage of OHCs in three turns (Apical, Middle, and Basal) of the cochlea. D. Quantification of SGCs stained by β-Tubulin III and DAPI. *P < 0.05; **P < 0.01 versus the control group. NE, noise exposure; OHCs, outer hair cells; SGCs, spiral ganglion cells.

cochlear sections were used for detection, focusing on the expression levels of the markers in IHCs and SGCs as the primary evaluation targets. As shown in **Figure 5A** and **5B**, an increase in 4-HNE expression was noted in IHCs the day following the first NE, persisting at elevated levels until day 14 compared to that in the control group. This expression was further amplified after a second NE. A similar pattern was observed in SGCs, indicating ROS accumulation in the cochlea (**Figure 6A**, **6B**). Moreover, we observed a continuous elevation in 8-OHdG expression within IHCs from the first NE, peaking 14 days after the second NE. This

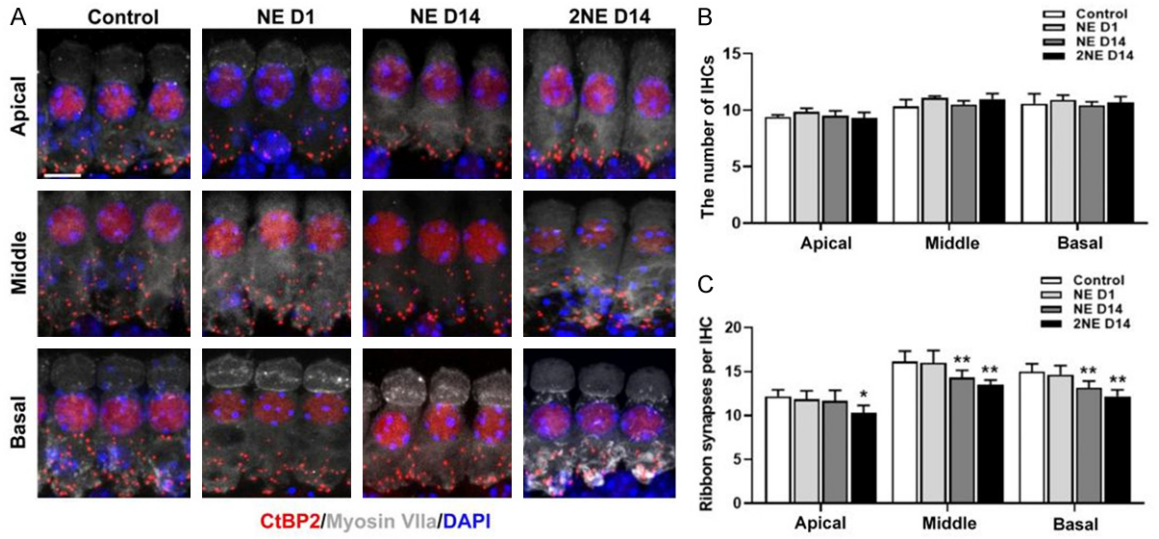

Figure 3. Noise exposure (NE) leads to the loss of ribbon synapses (RS). A. Representative images of immunolabeled RS (red) in IHCs with Myosin VIIIa staining (gray) after NE in the different groups. Scale bar = 5 μm. B. IHCs survival percentage in three turns (Apical, Middle, and Basal) of the cochlea. C. Quantification of RS stained by CtBP2. *P < 0.05; **P < 0.01 versus the control group. NE, noise exposure; RS, ribbon synapses; IHCs, inner hair cells.

trend was also mirrored in SGCs (**Figures 5A**, **5C**, **6A**, and **6C**). Given the predominant localization of 8-OHdG in the cytoplasm, we infer that the oxidative damage primarily occurs in mitochondrial DNA (mtDNA).

Collectively, our findings describe changes in oxidative stress levels in the cochlea at various time points post-NE. Specifically, ROS production in IHCs and SGCs increases immediately following NE and, despite a downward trend over time, remains elevated until day 14 post-exposure. Secondary NE administration further increases ROS accumulation. However, oxidative damage to mtDNA accumulates continuously and persists in the cochlea after NE.

Discussion

As the most common environmental factor in daily life, NE may cause two different effects on hearing: the hidden hearing loss manifested as TTS and the NIHL manifested as PTS [24, 25]. A previous study showed that TTS recovery does not necessarily imply a complete restoration of the auditory system, as it can leave behind synaptic damage and temporary demyelination of auditory nerve fibers (ANFs). Since short-term exposure to moderate noise can lead to TTS whereas repeated NE may result in PTS [26-28], mice were exposed to single or repeated white noise in our study to verify the development mechanisms of NIHL. Our findings align with previous research [29], demonstrating significant increases in hearing thresholds across all frequencies on post-exposure day 1, with a substantial recovery by day 14. This recovery might be attributable to the restoration of the interaction between stereocilia and the tectorial membrane on OHCs, as suggested by earlier studies [30]. However, despite apparent recovery in mice with TTS, there was a prolonged latency and decreased ABR wave I amplitude, indicative of synaptic function impairment. Moreover, neither hearing thresholds nor the alteration of ABR wave I failed to return to normal levels when the mice were exposed to secondary NE, suggesting further cochlear damage.

It is well known that there are two types of HCs in the cochlea: OHCs, which amplify and modulate sound, and IHCs, which form synaptic connections with type I ANFs, converting acoustic signals into electrical signals for brain transmission [31, 32]. Impairment of any HC type can lead to decreased sound sensitivity, clarity, and recognition. Several experiments have shown that pathological changes in synapses between IHCs and ANFs precede damage to HCs and SGCs in multiple models of hearing loss [33-35]. The RS is an electron-dense structure surrounded by synaptic vesicles that

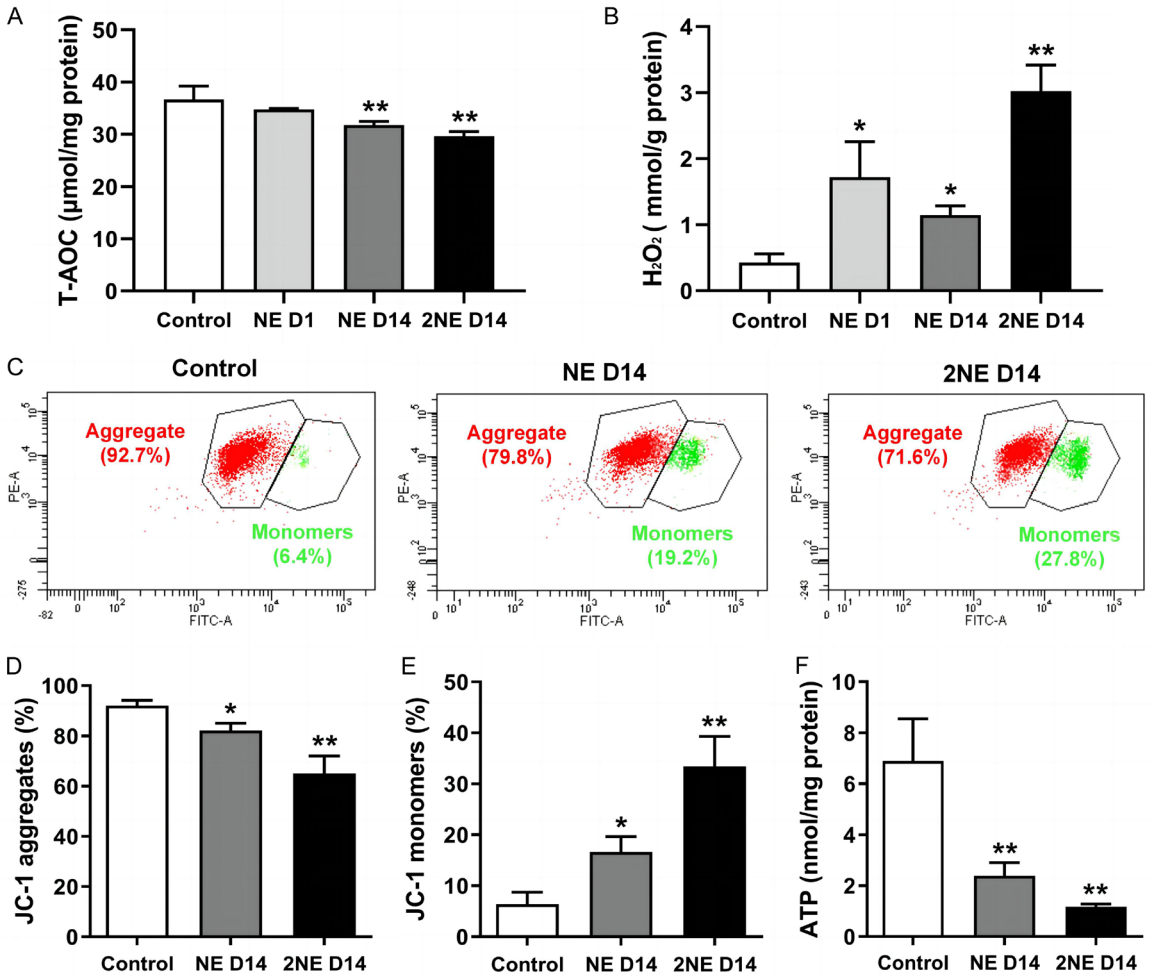

Figure 4. NE had an effect on mitochondrial function in the cochlea. A. Changes of T-AOC in the cochlea after NE of different time points. B. The accumulation of H_2O_2 in the cochlea after NE of different time points. C. Changes in MMP were detected by the JC-1 kit under flow cytometry. D. Percentage of JC-1 aggregates cells in the different time points after NE. E. Percentage of JC-1 monomer cells in the different time points after NE. F. The levels of ATP significantly decreased as the NE frequency increased. *P < 0.05; **P < 0.01 versus the control group. NE, noise exposure; MMP, mitochondrial membrane potential.

coordinate the fusion of presynaptic vesicles with the transport of information to the post-synaptic vesicles [36]. In auditory processing, the stress signal induced by acoustic vibration is converted into an electrical signal, with RS encoding the microphonics potential through various channels and glutamate exocrine mechanisms [37, 38]. Thus, different types and intensities of destructive NE result in the spatiotemporal distribution and dysfunction of RS. Given that the amplitude of ABR wave I relates closely to synaptic function, and considering that our results showed a decrease in the amplitude of ABR wave I, we further quantified the number of HCs, SGCs, and RS to investigate assess NE's impact on cochlear morphology. Our results indicated that NE did not alter the morphology or number of IHCs or SGCs. However, a significant reduction in RS was noted in the middle and basal turns of the membrane on post-exposure day 14 after the first NE, becoming more pronounced after the second NE. Additionally, the absence of OHCs was also observed in these turns following the second NE. These results strongly suggest that RS are more sensitive to noise stimulation in the cochlea than HCs and SGCs, and even moderate NE can lead to auditory dysfunction. It is worth mentioning that we found a loss of RS on post-exposure day 14, not day 1, indicating that RS absence does not occur immediately after NE.

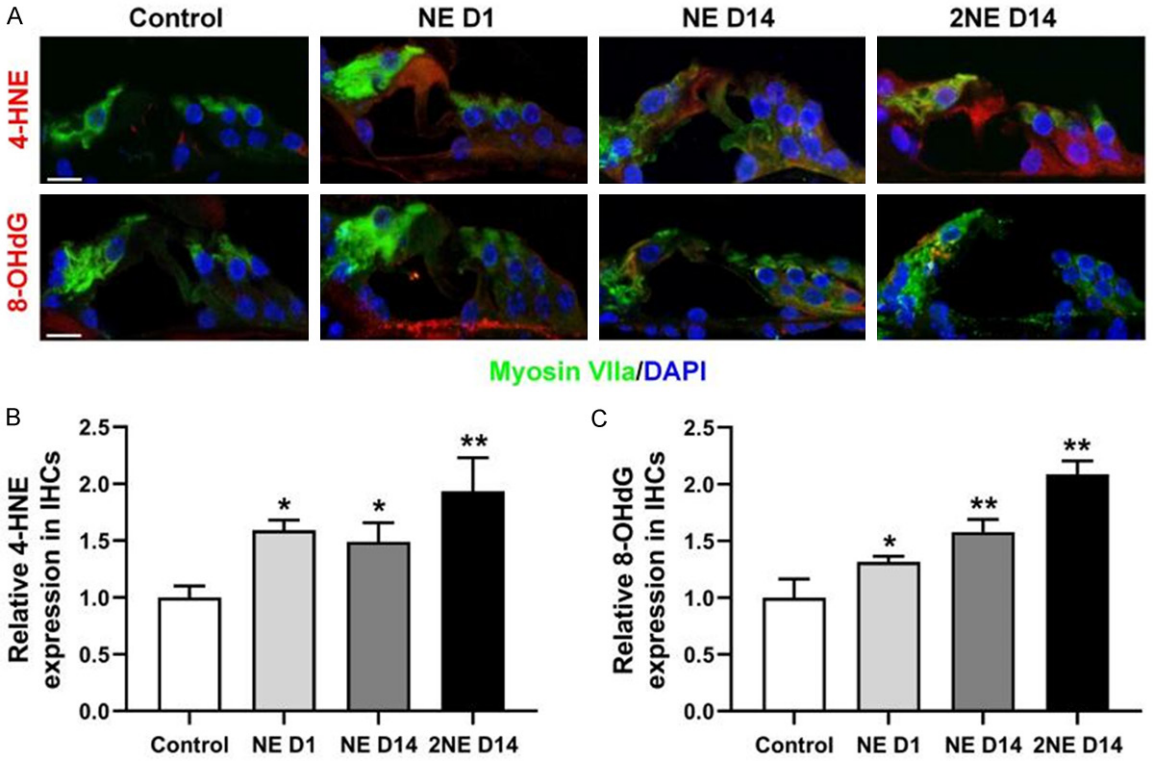

Figure 5. Noise exposure (NE) induced oxidative stress in the inner hair cells (IHCs). A. Representative images of the expression of 4-HNE (red) and 8-OHdG (red) on IHCs in different groups following immunohistochemical staining of frozen cochlea sections. Scale bars = 10 μm. B. Relative 4-HNE expression in IHCs of different time points after NE. C. Relative 8-OHdG expression in IHCs of different time points after NE. *P < 0.05; **P < 0.01 versus the control group. NE, noise exposure; IHCs, inner hair cells.

Among the various mechanisms implicated in NIHL, the oxidative stress theory is one of the most extensively discussed. Mitochondria, as primary sites of energy conversion, are crucial not only for ATP production but also for controlling programmed cell death by regulating membrane potentials. Under continuous or intense NE, the ability of IHCs to consistently renew synaptic vesicles for efficient neural signal transmission largely depends on a continuous mitochondrial energy supply. The density of mitochondria at neural axon terminals is crucial for maintaining RS numbers [39-41]. In D-galactose-induced aging mice cochlea, reduced ATP production due to mitochondrial oxidative damage led to progressive degeneration and dysfunction of RS. Furthermore, a dynamic correlation between synaptic changes and ATP levels has been observed. Consequently, we focused on changes in mitochondrial function indicators to identify possible mechanisms of noise-induced synaptic damage [42]. In our study, T-AOC decreased with the accumulation of H_2O_2 in the cochlea, indicating oxidative stress happens the moment after NE. Additionally, we observed a decrease in ATP and MMP levels. Normal MMP is essential for sustaining oxidative phosphorylation and ATP production [43]. Delayed clearance of ROS can lead to abnormal MMP, disrupting mitochondrial metabolism. This was similarly observed in vitro studies of the Corti apparatus by the House Ear Institute, where cells exposed to H_2O_2 aged prematurely compared to untreated cells. They found that alongside MMP reduction, parts of the mitochondrial structure were damaged [44]. Therefore, it is plausible to hypothesize that oxidative stress following NE results in mitochondrial dysfunction, increasing inner ear cells' susceptibility to oxidative damage.

As a by-product of mitochondrial respiration, ROS play a crucial role in cell signaling and oxidative homeostasis, integral to cellular energy metabolism [45]. Recognized as a key mediator of HCs damage, ROS affects multiple intra-

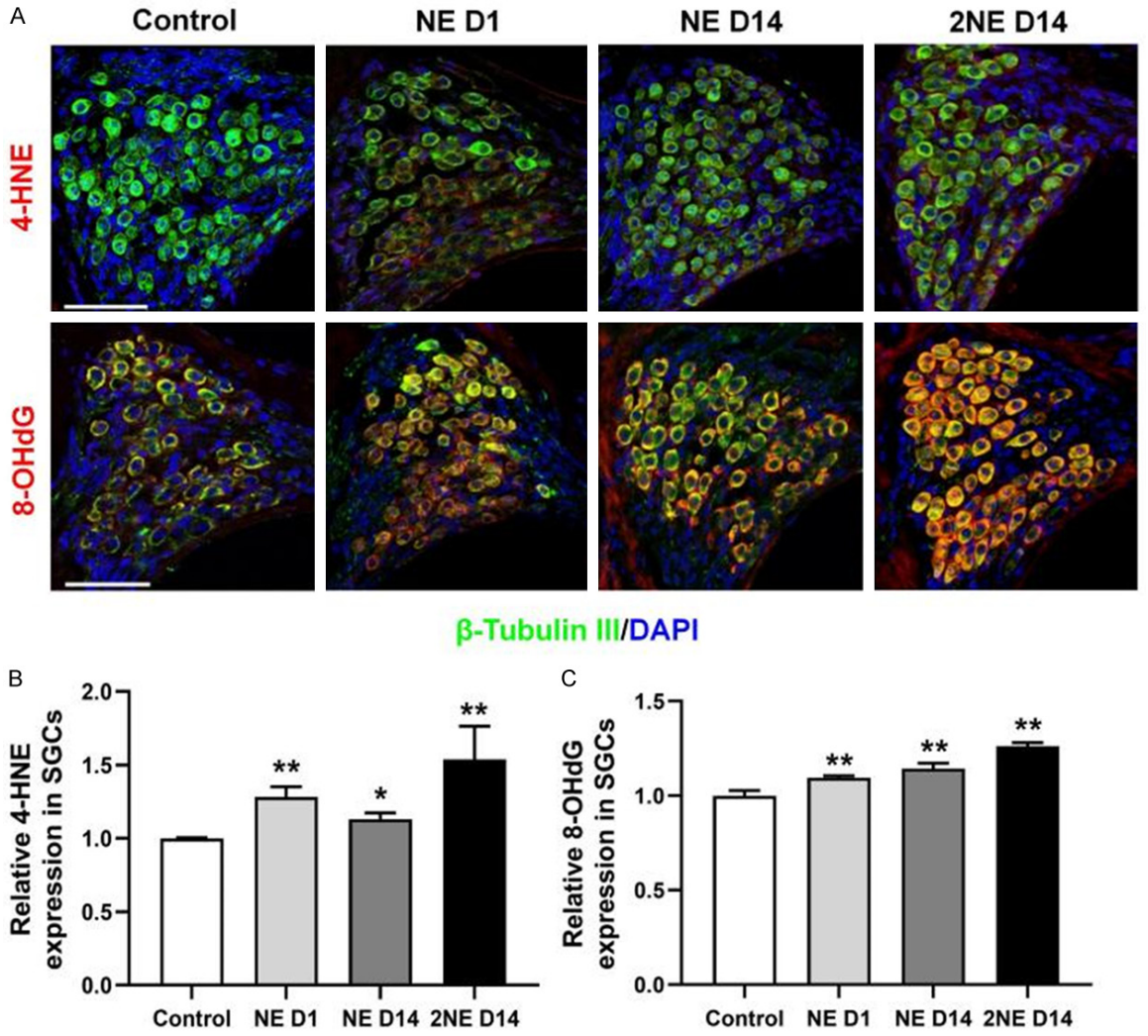

Figure 6. Noise exposure (NE) induced oxidative stress in the spiral ganglion cells (SGCs). A. Representative images of the expression of 4-HNE (red) and 8-OHdG (red) on SGCs in different groups following immunohistochemical staining of frozen cochlea sections. Scale bar = 40 μm. B. Relative 4-HNE expression in SGCs of different time points after NE. C. Relative 8-OHdG expression in SGCs of different time points after NE. *P < 0.05; **P < 0.01 versus the control group. NE, noise exposure; SGCs, spiral ganglion cells.

cellular processes by reacting with biological macromolecules such as DNA, proteins, cellular membrane molecules, and lipids [46, 47]. ROS production has been detected in cochleas exposed to noise and ototoxic drugs [48-50]. To further investigate the mechanism underlying mitochondrial dysfunction, we assessed the accumulation of ROS and oxidative damage in the cochlea. Through 4-HNE staining, we observed ROS production in the cochlea the day following NE. While ROS levels decreased after two weeks, they remained elevated compared to those in the control group, suggesting persistent ROS accumulation up to 14 days, with levels rising again upon secondary NE. Furthermore, 8-OHdG staining, predominantly in the cytoplasm of HCs and SGCs, where mitochondria are located, indicates oxidative damage to mtDNA. The intensity of 8-OHdG staining was exacerbated by secondary NE, supporting the notion that NE leads to progressive oxidative damage to mtDNA. The above results suggest that the generation and persistence of ROS is an early event in the process of noise-induced cochlear damage. It is this prolonged oxidative stress that leads to oxidative damage to mtDNA, inducing mitochondrial progressive dysfunction.

Conclusion

Our study presents an analysis of the auditory and pathological characteristics of NIHL in mice. We establish a link between mitochondrial dysfunction and synaptic damage, unveiling potential mechanisms of NIHL. Our findings confirm that noise-induced ROS production and its persistence contribute to oxidative mtDNA damage, and the resulting mitochondrial dysfunction further causes the accumulation of ROS. It is this vicious cycle that leads to the early onset of NIHL. These insights provide a theoretical foundation for early prevention and treatment strategies.

Acknowledgements

The work was supported by the National Natural Science Foundation of China (Nos. 82171132 and 81830030), and the Beijing Municipal Administration of Hospitals' Youth Programme (Code: QML20200107).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Zheng-De Du and Shu-Sheng Gong, Department of Otolaryngology Head and Neck Surgery, Beijing Friendship Hospital, Capital Medical University, No. 95, Yong'an Road, Xicheng District, Beijing 100050, China. E-mail: duzhengde@ccmu.edu.cn (ZDD); gongss@ccmu.edu.cn (SSG)

References

[1] Chadha S, Kamenov K and Cieza A. The world report on hearing, 2021. Bull World Health Organ 2021; 99: 242-242A.

[2] GBD 2019 Hearing Loss Collaborators. Hearing loss prevalence and years lived with disability, 1990-2019: findings from the Global Burden of Disease Study 2019. Lancet 2021; 397: 996-1009.

[3] Wang Y, Hirose K and Liberman MC. Dynamics of noise-induced cellular injury and repair in the mouse cochlea. J Assoc Res Otolaryngol 2002; 3: 248-268.

[4] Cui Y, Sun GW, Yamashita D, Kanzaki S, Matsunaga T, Fujii M, Kaga K and Ogawa K. Acoustic overstimulation-induced apoptosis in fibrocytes of the cochlear spiral limbus of mice. Eur Arch Otorhinolaryngol 2011; 268: 973-978.

[5] Shi L, Chang Y, Li X, Aiken S, Liu L and Wang J. Cochlear synaptopathy and noise-induced hidden hearing loss. Neural Plast 2016; 2016: 6143164.

[6] Canlon B. The effect of acoustic trauma on the tectorial membrane, stereocilia, and hearing sensitivity: possible mechanisms underlying damage, recovery, and protection. Scand Audiol Suppl 1988; 27: 1-45.

[7] Kim J, Xia A, Grillet N, Applegate BE and Oghalai JS. Osmotic stabilization prevents cochlear synaptopathy after blast trauma. Proc Natl Acad Sci U S A 2018; 115: E4853-E4860.

[8] Liochev SI. Reactive oxygen species and the free radical theory of aging. Free Radic Biol Med 2013; 60: 1-4.

[9] Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417: 1-13.

[10] Benkafadar N, François F, Affortit C, Casas F, Ceccato JC, Menardo J, Venail F, Malfroy-Camine B, Puel JL and Wang J. ROS-induced activation of DNA damage responses drives senescence-like state in postmitotic cochlear cells: implication for hearing preservation. Mol Neurobiol 2019; 56: 5950-5969.

[11] Wu P, Wu X, Zhang C, Chen X, Huang Y and Li H. Hair cell protection from ototoxic drugs. Neural Plast 2021; 2021: 4909237.

[12] Ohlemiller KK, Wright JS and Dugan LL. Early elevation of cochlear reactive oxygen species following noise exposure. Audiol Neurootol 1999; 4: 229-236.

[13] Liberman MC and Kujawa SG. Cochlear synaptopathy in acquired sensorineural hearing loss: manifestations and mechanisms. Hear Res 2017; 349: 138-147.

[14] Bramhall N, Beach EF, Epp B, Le Prell CG, Lopez-Poveda EA, Plack CJ, Schaette R, Verhulst S and Canlon B. The search for noise-induced cochlear synaptopathy in humans: mission impossible? Hear Res 2019; 377: 88-103.

[15] Du ZD, Han SG, Qu TF, Guo B, Yu SK, Wei W, Feng S, Liu K and Gong SS. Age-related insult of cochlear ribbon synapses: an early-onset contributor to D-galactose-induced aging in mice. Neurochem Int 2020; 133: 104649.

[16] Jeng JY, Ceriani F, Olt J, Brown SDM, Holley MC, Bowl MR, Johnson SL and Marcotti W. Pathophysiological changes in inner hair cell ribbon synapses in the ageing mammalian cochlea. J Physiol 2020; 598: 4339-4355.

[17] Luo Y, Qu T, Song Q, Qi Y, Yu S, Gong S, Liu K and Jiang X. Repeated moderate sound exposure causes accumulated trauma to cochlear ribbon synapses in mice. Neuroscience 2020; 429: 173-184.

[18] Feng S, Yang L, Hui L, Luo Y, Du Z, Xiong W, Liu K and Jiang X. Long-term exposure to low-intensity environmental noise aggravates age-related hearing loss via disruption of cochlear

ribbon synapses. Am J Transl Res 2020; 12: 3674-3687.
[19] Zhang L, Du Z, He L, Liang W, Liu K and Gong S. ROS-induced oxidative damage and mitochondrial dysfunction mediated by inhibition of SIRT3 in cultured cochlear cells. Neural Plast 2022; 2022: 5567174.
[20] Fetoni AR, Paciello F, Rolesi R, Paludetti G and Troiani D. Targeting dysregulation of redox homeostasis in noise-induced hearing loss: oxidative stress and ROS signaling. Free Radic Biol Med 2019; 135: 46-59.
[21] Kim YR, Baek JI, Kim SH, Kim MA, Lee B, Ryu N, Kim KH, Choi DG, Kim HM, Murphy MP, Macpherson G, Choo YS, Bok J, Lee KY, Park JW and Kim UK. Therapeutic potential of the mitochondria-targeted antioxidant mitoQ in mitochondrial-ROS induced sensorineural hearing loss caused by IDH2 deficiency. Redox Biol 2019; 20: 544-555.
[22] Lobarinas E, Spankovich C and Le Prell CG. Evidence of "hidden hearing loss" following noise exposures that produce robust TTS and ABR wave-I amplitude reductions. Hear Res 2017; 349: 155-163.
[23] Chen JW, Ma PW, Yuan H, Wang WL, Lu PH, Ding XR, Lun YQ, Yang Q and Lu LJ. mito-TEMPO attenuates oxidative stress and mitochondrial dysfunction in noise-induced hearing loss *via* maintaining TFAM-mtDNA interaction and mitochondrial biogenesis. Front Cell Neurosci 2022; 16: 803718.
[24] Liberman MC. Hidden hearing loss. Sci Am 2015; 313: 48-53.
[25] C Kohrman D, Wan G, Cassinotti L and Corfas G. Hidden hearing loss: a disorder with multiple etiologies and mechanisms. Cold Spring Harb Perspect Med 2020; 10: a035493.
[26] Shi L, Liu L, He T, Guo X, Yu Z, Yin S and Wang J. Ribbon synapse plasticity in the cochleae of guinea pigs after noise-induced silent damage. PLoS One 2013; 8: e81566.
[27] Fernandez KA, Jeffers PW, Lall K, Liberman MC and Kujawa SG. Aging after noise exposure: acceleration of cochlear synaptopathy in "recovered" ears. J Neurosci 2015; 35: 7509-7520.
[28] Song F, Gan B, Wang N, Wang Z and Xu AT. Hidden hearing loss is associated with loss of ribbon synapses of cochlea inner hair cells. Biosci Rep 2021; 41: BSR20201637.
[29] Liang W, Zhao C, Chen Z, Yang Z, Liu K and Gong S. Sirtuin-3 protects cochlear hair cells against noise-induced damage via the superoxide dismutase 2/reactive oxygen species signaling pathway. Front Cell Dev Biol 2021; 9: 766512.
[30] Nordmann AS, Bohne BA and Harding GW. Histopathological differences between temporary and permanent threshold shift. Hear Res 2000; 139: 13-30.
[31] Salvi R, Sun W, Ding D, Chen GD, Lobarinas E, Wang J, Radziwon K and Auerbach BD. Inner hair cell loss disrupts hearing and cochlear function leading to sensory deprivation and enhanced central auditory gain. Front Neurosci 2017; 10: 621.
[32] Shubina-Oleinik O, Nist-Lund C, French C, Rockowitz S, Shearer AE and Holt JR. Dual-vector gene therapy restores cochlear amplification and auditory sensitivity in a mouse model of DFNB16 hearing loss. Sci Adv 2021; 7: eabi7629.
[33] Sergeyenko Y, Lall K, Liberman MC and Kujawa SG. Age-related cochlear synaptopathy: an early-onset contributor to auditory functional decline. J Neurosci 2013; 33: 13686-13694.
[34] Liu K, Jiang X, Shi C, Shi L, Yang B, Shi L, Xu Y, Yang W and Yang S. Cochlear inner hair cell ribbon synapse is the primary target of ototoxic aminoglycoside stimuli. Mol Neurobiol 2013; 48: 647-654.
[35] Fernandez KA, Guo D, Micucci S, De Gruttola V, Liberman MC and Kujawa SG. Noise-induced cochlear synaptopathy with and without sensory cell loss. Neuroscience 2020; 427: 43-57.
[36] Wan G, Gómez-Casati ME, Gigliello AR, Liberman MC and Corfas G. Neurotrophin-3 regulates ribbon synapse density in the cochlea and induces synapse regeneration after acoustic trauma. Elife 2014; 3: e03564.
[37] Zhou Y, Zheng H, Ruan F, Chen X, Zheng G, Kang M, Zhang Q and Sun X. Hydrogen-rich saline alleviates experimental noise-induced hearing loss in guinea pigs. Neuroscience 2012; 209: 47-53.
[38] Suzuki J, Corfas G and Liberman MC. Round-window delivery of neurotrophin 3 regenerates cochlear synapses after acoustic overexposure. Sci Rep 2016; 6: 24907.
[39] Stowers RS, Megeath LJ, Górska-Andrzejak J, Meinertzhagen IA and Schwarz TL. Axonal transport of mitochondria to synapses depends on milton, a novel drosophila protein. Neuron 2002; 36: 1063-1077.
[40] Li Z, Okamoto K, Hayashi Y and Sheng M. The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell 2004; 119: 873-887.
[41] Kang JS, Tian JH, Pan PY, Zald P, Li C, Deng C and Sheng ZH. Docking of axonal mitochondria by syntaphilin controls their mobility and affects short-term facilitation. Cell 2008; 132: 137-148.
[42] Guo B, Guo Q, Wang Z, Shao JB, Liu K, Du ZD and Gong SS. D-galactose-induced oxidative stress and mitochondrial dysfunction in the cochlear basilar membrane: an in vitro aging model. Biogerontology 2020; 21: 311-323.
[43] Du ZD, Yu S, Qi Y, Qu TF, He L, Wei W, Liu K and Gong SS. NADPH oxidase inhibitor apocynin

decreases mitochondrial dysfunction and apoptosis in the ventral cochlear nucleus of d-galactose-induced aging model in rats. Neurochem Int 2019; 124: 31-40.
[44] Kamogashira T, Hayashi K, Fujimoto C, Iwasaki S and Yamasoba T. Functionally and morphologically damaged mitochondria observed in auditory cells under senescence-inducing stress. NPJ Aging Mech Dis 2017; 3: 2.
[45] Brand MD. Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. Free Radic Biol Med 2016; 100: 14-31.
[46] Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002; 82: 47-95.
[47] Juan CA, Pérez De La Lastra JM, Plou FJ and Pérez-Lebeña E. The chemistry of reactive oxygen species (ROS) revisited: outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. Int J Mol Sci 2021; 22: 4642.
[48] Kim HJ, Lee JH, Kim SJ, Oh GS, Moon HD, Kwon KB, Park C, Park BH, Lee HK, Chung SY, Park R and So HS. Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity. J Neurosci 2010; 30: 3933-3946.
[49] Vlajkovic SM, Chang H, Paek SY, Chi HH, Sreebhavan S, Telang RS, Tingle M, Housley GD and Thorne PR. Adenosine amine congener as a cochlear rescue agent. Biomed Res Int 2014; 2014: 841489.
[50] Kurabi A, Keithley EM, Housley GD, Ryan AF and Wong AC. Cellular mechanisms of noise-induced hearing loss. Hear Res 2017; 349: 129-137.