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Mitochondrial DNA common deletion increases susceptibility to noise-induced hearing loss in a mimetic aging rat model

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ABSTRACT

Noise-induced hearing loss (NIHL) is an important occupational health hazard. However, susceptibility to NIHL remains poorly understood. The present study was designed to investigate whether mitochondrial DNA common deletion (CD) increases the susceptibility of individuals to NIHL. A mimetic aging rat model harboring increased CD in the inner ear was established by chronic D-galactose administration, and the synergic effect of CD and noise on hearing sensitivity was assessed. We determined that although developed the same magnitude of temporary threshold shifts and hair cell loss, the D-galactose treated rats with increased CD in the inner ear exhibited a longer hearing recovery process and experienced higher permanent hearing threshold shifts at high frequencies than the saline-treated control rats. Greater supporting cell damage and stria vascularis ultrastructural changes were observed in D-galactose treated rats three weeks after recovery. The results suggested that the elevated CD in the inner ear could increase an individual's susceptibility to NIHL, which likely through a reduction in the self-repairing capability within the cochlea after acoustic injury.

1. Introduction

Noise-induced hearing loss (NIHL) is one of the most important occupational hazards and has become the second most frequent form of hearing impairment [1]. In developing countries, millions of people are exposed to damaging levels of noise in their work environment and are at risk of developing NIHL. Acoustic overstimulation can produce temporary threshold shifts (TTSs) and permanent threshold shifts (PTSs) in auditory perception. The severity of hearing loss is mainly determined by the intensity, duration, and frequency range of the noise stimulus. However, it has long been known that individuals have different susceptibilities to NIHL [2]. When exposed to the same level of noise, some individuals develop significant hearing loss, whereas others experience no changes. The molecular mechanisms underlying the differences in different individuals' susceptibilities to NIHL remain obscure.

Many studies have suggested that the incidence of NIHL is markedly due to the interactions of environmental factors, individual factors, and genetic backgrounds [3]. Many environmental/occupational chemical contaminants, including solvents, metals, asphyxiants, and pesticides, have a synergistic effect with noise [4]. Lataye et al. found that rats exposed to styrene and noise simultaneously suffer more hearing loss and greater hair cell damage than those exposed to each agent alone [5]. In addition, individual factors, such as age, nutritional status and cardiovascular problems, have also been proposed as risk factors that can potentate NIHL [6]. For example, Wu et al. observed that rats with diabetes were prone to developing more severe NIHL than normal rats when exposed to the same level of noise [7]. In addition to environmental factors and the individual conditions that are associated with NIHL risk, there is accumulating evidence suggesting a possible genetic role. Erway et al. observed that mouse strains with the *Ahl* gene, which is responsible for age-related hearing loss, exhibit progressive hearing loss at an early age and are markedly more susceptible to noise than those without the *Ahl* gene [8]. Some recent studies have proposed mitochondrial gene mutation as a contributor to NIHL because the feature of mitochondrial DNA (mtDNA) deafness is that the same mutation produces a clinical phenotype that varies considerably [3,9]. However, knowledge of the mitochondrial gene responsible for NIHL remains limited.

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The mtDNA 4977-bp deletion (also termed "common deletion", CD) in humans, which corresponds to the mtDNA 4834-bp deletion in rats, has been found to be commonly accumulated in inner ear tissues with aging and associated with presbycusis [10,11]. We previously found that the inner ear of a mimetic aging rat model induced by the chronic injection of D-galactose (D-gal) showed a high presence of CD [9,12,13]. Furthermore, we also found that rats with a sub-damaging level of CD in the inner ear exhibited no hearing loss but are much more sensitive to aminoglycoside ototoxicity than control rats [9]. These observations suggested that CD may serve as a predisposing factor that can markedly enhance the sensitivity of the inner ear to ototoxic stresses. Additionally, it has been found that noise exposure can cause oxidative DNA damage in the cochlea [14], and a relationship between CD and long-term noise exposure was recently observed [15], indicating that there is likely an interaction effect between CD and noise exposure. Moreover, mtDNA deletions are always heteroplasmic [16]. Because the level of heteroplasmy varies between individuals, the synergistic effect of mtDNA deletions with noise may differ among individuals. Therefore, the CD may serve as a candidate gene mutant responsible for the susceptibility of an individual to NIHL.

The present study was designed to investigate this hypothesis using a mimetic aging rat model with increased cochlear CD induced by D-gal, which has been well established [9,12,13]. A chronic noise exposure condition was chosen to mimic occupational noise exposure and to maximize the interaction of noise and CD [15].

2. Materials and methods

2.1. Animal treatment

Female SD rats aged 1 month were purchased from the Experimental Animal Center of Tongji Medical College and caged in a low-noise environment (<60 dB SPL) with a 12-h light/12-h dark cycle. All of the animal procedures were approved by the Institutional Animal Care and Use Committee of Tongji Medical College. After acclimation for 2 weeks, 72 rats were randomly attributed into four groups: control group, D-gal group, noise group and D-gal + noise group. The rats in the D-gal and D-gal + noise groups were subcutaneously administered 500 mg/kg D-gal daily for 8 weeks (termed "D-gal treated rats"). The rats in the control and noise groups were given the same volume of vehicle (normal saline) on the same schedule (termed "saline-treated rats"). After a 10-day break, the rats in the noise and D-gal + noise groups were then exposed to 110-dB SPL continuous white noise at a rate of 6 h per day for 20 consecutive days. The rats in the control and D-gal groups were sham exposed with the same schedule.

2.2. Noise exposure

The noise exposure was performed in a soundproof chamber. The acoustic stimulus was generated by a custom-made white noise generator. The sound signals were amplified by a Sony Power Amplifier (Sony, Japan) and fed to a loudspeaker located approximately 15 cm in front of the subjects' cage. The animals were awake and unrestrained in a 21 × 21 × 11-cm wire cage mounted on a rotating platform in the sound field. The cage was rotated very slowly during the exposure period to achieve a homogenous sound field. The overall noise level was measured and monitored at four positions within the cage at a level approximately equal to the level of the animal's ear using a TES135 sound level meter (TES, China). The sound pressure varied less than 2 dB within the space available to each animal. During the exposure, the animals had free access to food and water.

2.3. Evaluation of auditory function

Auditory brainstem response (ABR) was examined before drug administration, before noise exposure and 1, 7, 14, 21 days after noise exposure. Rats were anesthetized with a mixture of ketamine (30 mg/kg i.p.) and chlorpromazine (15 mg/kg, i.p.), and the body temperature were maintained at 37 °C using a heating pad. ABR response was recorded using a TDT system III (Tucker-Davis Technologies, Alachua, FL,USA). The tone burst stimuli at 4, 8, 16, 24, and 32 kHz were generated and delivered into the rat external auditory canal through an electrostatic speaker with a 10-cm plastic tube. The evoked potentials were filtered between 100 and 3000 Hz and averaged 512 times. The lowest sound level that elicited a repeatable wave was considered as the threshold.

2.4. DNA preparation and quantitative RT-PCR

The mtDNA CD was determined 10 days post D-gal treatment and 21 days after noise exposure by quantitative RT-PCR as our previous report [12]. Membranous labyrinth tissues of cochlea were carefully dissected out and total DNA from each cochlea was extracted using the Genomic DNA Purification Kit (Tiangen, China). The TaqMan RT-PCR assay primers and probes for mtDNA D-loop and CD were previously described [17]. The PCR amplification was performed using a StepOnePlus™ Real-time PCR system (Applied Biosystems, CA, USA). The cycling conditions include one cycle at 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The D-loop copy number was used as a measure of the total amount of mtDNA in each sample. The relative amount of CD was calculated by using $2^{-\Delta\Delta Ct}$ method. Agarose gel electrophoresis (AGE) and DNA sequencing were performed to test the specificity and integrity of the PCR product.

2.5. Cochlear tissue preparation and pathological observation

As we previously described [18], animals were anesthetized and perfused transcardially with 4% formaldehyde in PBS. The cochleae were carefully dissected from the temporal bone and kept in the same fixative at 4 °C overnight. After decalcification with 10% sodium EDTA and further post-fixation in 1% osmium, the cochleae were dehydrated through a graded ethanol and acetone series, and embedded in Epon 812. Sections with a thickness of 2 μm and 0.5 μm were cut for light microscopy observation and transmission electronic microscopy (TEM) examination, respectively.

For cochlear flat-surface preparations, the basilar membrane of the cochlea after fixation was carefully dissected out and rinsed in 0.3% Trion X-100 PBS for 30 min, and then stained with DAPI (Sigma, USA) for 20 min. After rinsing with PBS, the segments of the basilar membrane were mounted on glass slides and coverslipped. Hair cell counting was performed under a fluorescence microscope (400×) and a cochleogram was constructed.

2.6. Statistical analysis

Data are presented as the means ± SEM. Repeated-measures analysis of variance (ANOVA) was performed to test the difference of the hearing threshold shifts and a least significant difference (LSD) post hoc test was used to evaluate differences between groups. The difference in the CD and hair cell loss was assessed by one-way ANOVA. Differences with a P value < 0.05 were considered statistically significant.

3. Results

3.1. mtDNA CD determination and analysis

By 10 days after D-gal treatment, CD can be detected in all of the samples obtained from the D-gal treated rats and even in tissues from the saline-treated control rats by the quantitative real-time PCR. The CD level was significantly higher (approximately 1.6-fold) in the D-gal treated animals compared with the saline-treated animals ($P < 0.01$) (Fig. 1A). By 21 days after noise exposure, the CD level was again examined to evaluate the effect of noise on the CD level. No significant difference was observed between the noise and control groups ($P > 0.05$). In addition, the level of CD in the D-gal + noise group was similar that in the D-gal group ($P > 0.05$) (Fig. 1B). The size of the PCR products obtained with the CD primers was 81 bp, which is the same as that designed, and the sequences of the PCR products are listed to prove the presence of CD (Fig. 1C and D).

3.2. Hearing threshold shifts after D-gal injection and noise exposure

As previously reported [9,13], no significant hearing threshold changes were observed 10 days after D-gal treatment. This result indicated that all of these animals were suitable for our next NHIL experiment.

In the current experiment, the TTS or PTS was computed by subtracting the ABR threshold recorded before noise exposure from those measured at 1 day or 21 days post noise exposure [19]. After exposure to noise, all of the animals in the noise and D-gal + noise groups developed a large TTS (approximately 40–70 dB) at all of the frequencies tested; however, no significant differences in the TTS were found between the two groups ($P > 0.05$) (Fig. 2A). Three weeks after hearing recovery, a moderate PTS (approximately 20–40 dB) was observed. The PTS of the two groups were similar at the frequencies of 4, 8 and 16 kHz, but the PTS of the D-gal + noise group at 24 and 32 kHz was significantly higher than that of the noise group ($P < 0.05$) (Fig. 2A). The data indicated that CD in the inner ear did not potentate noise-induced TTS but rather deteriorated the PTS.

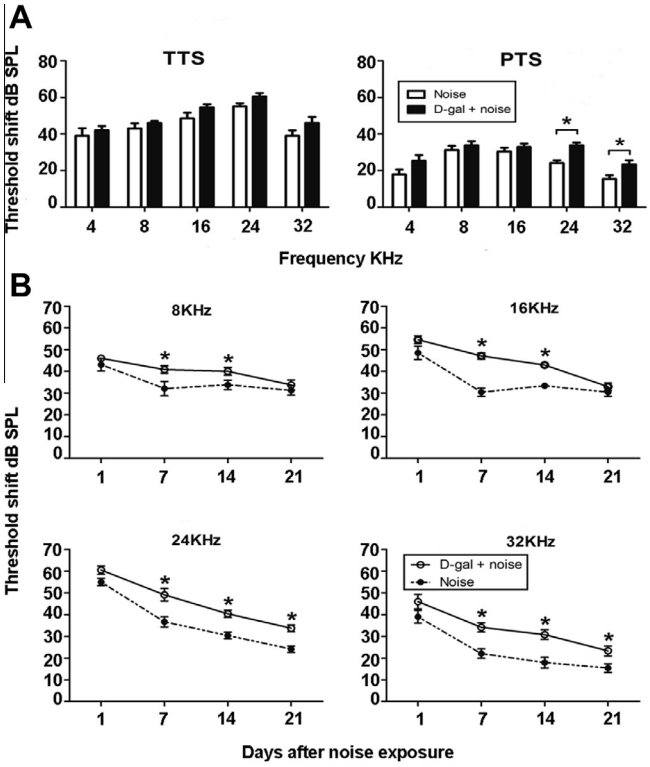

Fig. 2. (A) Noise-induced TTS and PTS at different frequencies. (B) Recovery of hearing sensitivity after noise exposure. $n = 12$ per group, $^*P < 0.05$ compared with the noise group in (A and B).

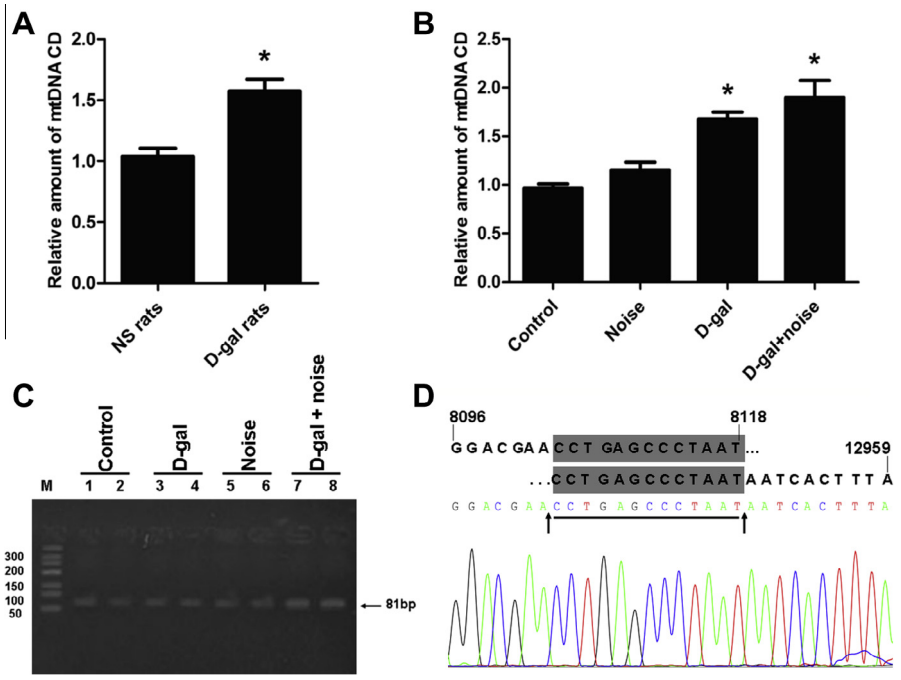

Fig. 1. (A) The relative amount of CD in the saline-treated rats (NS rats) and D-gal-treated rats (D-gal rats) 10 days after treatment. (B) The relative level of CD in the different groups 21 days after noise exposure. (C) PCR products obtained using mtDNA CD primers. The 81-bp product indicates the presence of CD. M = DNA marker. (D) Schematic diagram of CD. The bold black letters indicate the nucleotide sequences flanking the breakpoints of the deleted mtDNA, and the shaded letters indicate the direct repeats. The arrows point to the possible breakpoints. $n = 9$ per group, $^*P < 0.05$ compared with the NS rats in (A) and the control rats in (B).

3.3. Recovery of hearing sensitivity after noise exposure

It is known that a hearing function restoring process follows noise exposure [20]. After several hours or days of recovery, the TTS returned to the pre-exposure baseline or the PTS depending on the parameters of the acoustic overstimulation and the health conditions of the cochlea itself. Currently, we found that the TTS in the noise group recovered rapidly, whereas in the D-gal + noise group, the TTS recovery was significantly prolonged. As is shown in Fig. 2B, at 8 and 16 kHz, the TTS in the noise group rapidly recovered to a stable level seven days later; however, the TTS in the D-gal + noise group did not return to the same level until 21 days post noise exposure. At high frequencies (24 and 32 kHz), the hearing threshold shifts in the D-gal + noise group were even higher at all of the measured time points than those in the noise group. This finding suggested that CD delayed the recovery or repair processes within the cochlea due to noise-induced damage.

3.4. Hair cell loss

No or mild hair cell loss was observed in the control and D-gal groups. However, obvious hair cell loss was found in most of the rats in the noise and D-gal + noise groups after noise exposure. The loss of hair cells was observed mainly in the basal turn of the cochlea, and most lost cells were outer hair cells (OHC), particularly those in the first row. Inner hair cells (IHC) were intact. Hair cell counting showed that approximately 30–40% of the OHCs in noise and D-gal + noise groups were lost in the cochlear basal turn, and there was no significant difference between the two noise-exposure groups ($P > 0.05$) (Fig. 3).

3.5. Supporting cell damage after noise exposure

The outer space of Nuel (asterisk) and supporting cells were shown in the normal organ of Corti (Fig. 4A). Three weeks after recovery from noise damage, the outer space of Nuel in the noise group appeared normal, but large vacuoles could be observed in some of the Hensen cells (Fig. 4A, arrow). In contrast, in the D-gal + noise group, the outer space of Nuel completely disappeared, and the supporting cells appeared more swollen than those of the control and noise groups (Fig. 4A, arrowhead). The data indicated that the rats in the D-gal + noise group experienced greater supporting cell damage than those in the noise group after 21 days of recovery following noise exposure.

3.6. Altrastructural changes of stria vascularis

The stria vascularis (SV), an essential structure of the inner ear, contains abundant mitochondria. The normal ultrastructural features of the stria vascularis comprise three cell types: marginal, intermediate, and basal. The cells were arranged closely and the strial capillaries were open with normal endothelial cells. The marginal cells were rich in mitochondria; the shape and size of the mitochondria were normal (Fig. 4B). In the D-gal group, the ultrastructure features of the stria vascularis were similar to those of the control, but some of the mitochondria in the marginal cells were swollen with a reduced electron density in the matrix (arrowhead). In the noise group, the structure of the stria vascularis became swollen with enlarged intercellular spaces and decreased density. The intermediate cells exhibited shrunken and degenerative changes (star). The strial capillaries were enclosed and degenerated (arrow). The mitochondria in the marginal cells were swollen with cristae disruption. Greater SV damages could be found in the D-gal + noise group.

4. Discussion

Noise, as one of the most prevalent occupational ototoxic stresses, can cause TTS and/or PTS, which seriously influences the quality of an individual's daily life. A number of studies have ascribed noise-induced TTS and PTS to cochlear reversible and irreversible damage, respectively [20,21]. The present study provides us an opportunity to gain insights into the differences between these two types of damage, which may help explain the susceptibility to damage from noise exposure. Several types of reversible structural changes in the cochlea have been proposed as important contributors to TTS, including changes in the stereocilia bundle, the collapse of supporting cells, acute strial edema and the swelling of afferent nerve fibers [20]. However, it is thought that the most prominent pathological change that contributes to PTS is the loss of hair cells [20]. However, because noise can damage most of the cell populations in the cochlea, some of the other structural changes that cannot fully recover from the initial damage could also be attributed to the PTS [22]. In our experiment, we found that

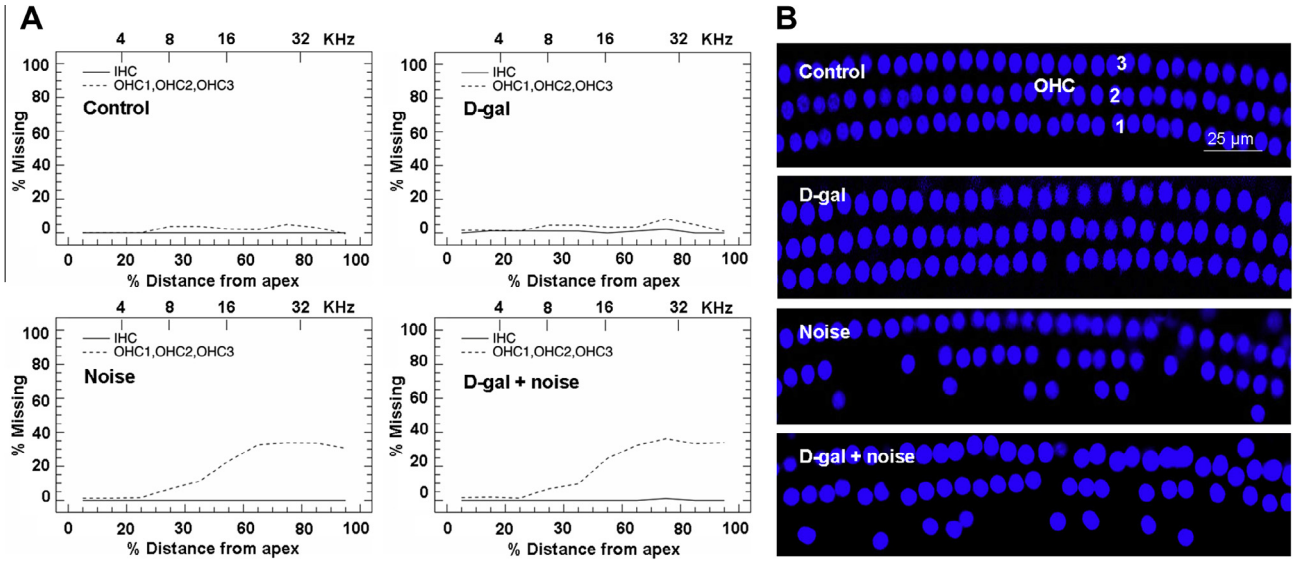

Fig. 3. (A) Mean cochleograms ($n = 5$ ears) of different groups showing the percentage of missing OHCs and IHCs as a function of the percent distance from the apex. (B) Examples of OHCs at a location related to 24 kHz (approximately 60% from the apex) in the different groups.

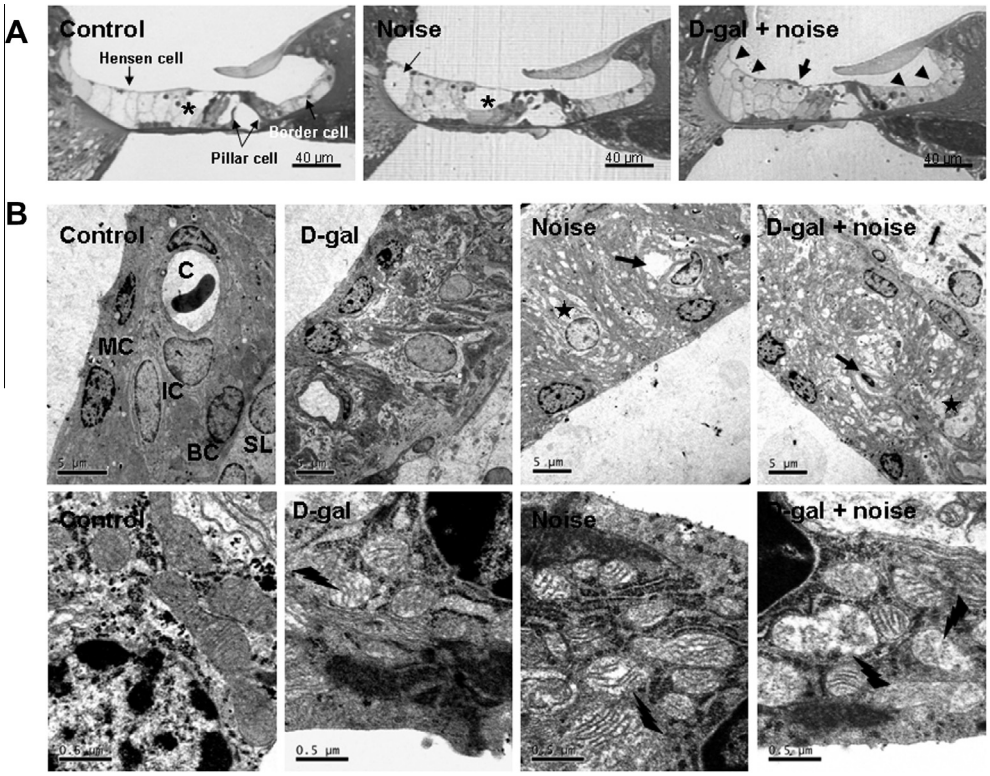

Fig. 4. (A) Representative images of organ of Corti from the upper basal turn of the cochlea 21 days post noise exposure illustrating the supporting cell damage after noise exposure. (B) TEM micrographs of the stria vascularis at the upper basal turn of the cochlea 21 days after noise exposure. The upper images show the structure of the stria vascularis in the different groups. The lower images show the ultrastructure of the mitochondria in the marginal cells. MC = marginal cell, IC = intermediate cell, BC = basal cell, C = capillary, SL = spiral ligament.

the two noise-exposure groups exhibited similar levels of TTS and hair cell loss but experienced different recovery times and PTSs at high frequencies, indicating that the difference may be mainly due to the cochlear repair process of some other structural injuries after acoustic trauma. This hypothesis was further confirmed by our results. After 21 days of recovery, the collapse of the outer space of Nuel and supporting cell swollen in the cochlea of the D-gal + noise group can still be observed, whereas in the noise group, the supporting cell damage was mostly repaired (Fig. 4A). Although we did not examine acute pathological changes following noise overstimulation, according to previous studies [20,21], these reversible structural changes should be responsible for the auditory functional reversibility. It is known that the region of mtDNA CD encodes several mitochondrial proteins that are essential for oxidative phosphorylation, such as ATPase subunit 6 and 8, ND3, ND4, ND5, and COXIII. Thus, the CD may result in defects the mitochondrial respiratory function, leading to cellular dysfunction and ion imbalance in the cochlea. The ultrastructural pathological changes observed in the stria vascularis that are critical for maintenance of the cochlear ionic environment provide direct evidence supporting this hypothesis (Fig. 4B). Given the prolonged hearing recovery process in the D-gal + noise group, we can speculate that the pathological process induced by CD in the inner ear together with the effect of noise can significantly reduce, or even abolish, the capability of self-repair in the cochlea after acoustic injury, which may contribute to the susceptibility to noise-induced cochlear damage. This may explain why all of the noise-exposed rats developed the same level of TTS but ultimately showed different PTSs, particularly at high frequencies.

It is interesting to note that, even though large threshold shifts were observed at low frequencies (4, 8 and 16 kHz) in the two noise-exposure groups, only a few hair cells were lost in the low-frequency-related region of the cochlea (Figs. 2 and 3). Previous studies have indicated that the relationship between noise-induced functional impairment and loss of hair cells in rats is a lack of correlation [23,24]. Chen et al. examined the rat CAP threshold elevation and hair cell loss four weeks post noise exposure and found that there was no hair cell loss in the apical turn, even when a 60-dB CAP threshold elevation was induced. In the middle turn, significant hair cell loss was not observed until the CAP threshold elevation exceeded approximately 40–50 dB. In contrast, in the basal turn, OHC loss was observed in almost all of the noise-exposed rats, even without detectable NIHL in some cases. In addition, these studies indicated that NIHL may stem from hair cell injury or other cochlear impairments besides hair cell death [24], which is consistent with the results of the present study. Because the cochlea is a well-designed organ, the marked changes observed in the supporting cell architecture would be expected to change the cochlear micromechanics and thus the hearing sensitivity [20]. Moreover, we speculate that the increased CD in the hair cell or supporting cells may significantly impair the auditory function of surviving cells and makes it difficult to recover from or repair the injury. A good correlation between NIHL and non-lethal impairments of surviving hair cells was recently illustrated [23,25], and the fact that hair cells may survive without auditory function was confirmed. This may explain why NIHL is detected without hair cell loss in some regions of the cochlea and why rats in the D-gal + noise group showed a prolonged hearing recovery process.

In summary, in the current experiment, we demonstrated that the elevated level of mtDNA CD in the inner ear can increase an individual's susceptibility to NIHL, likely through a reduction in the self-repairing capability within the cochlea after acoustic overstimulation. Because the CD accumulates in the cochlea with aging

[10], this may partly explain why the elderly are more sensitive to NIHL [26]. However, to clarify this issue, further studies are warranted.

Acknowledgments

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