Changes in Guinea Pig Cochlear Hair Cells after Sound Conditioning and Noise Exposure

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Abstract: Changes in Guinea Pig Cochlear Hair Cells after Sound Conditioning and Noise Exposure: Hongyan Zuo, *et al.* Department of Occupational Hygiene, Institute of Health and Environmental Medicine of Tianjin, PR China—

Sound conditioning has reduced noise-induced hearing loss in experimental mammalian animals and in clinical observation. Forty guinea pigs were grouped as: A, control; B, conditioning noise exposure group; C, high level noise exposure group; and D, conditioning noise exposure followed by a high level noise exposure group. Auditory brainstem response thresholds were measured. The cochlear sensory epithelia surface was observed microscopically. Calmodulin, F-actin and heat shock protein 70 (HSP70) in hair cells were immunohistochemically stained. The intracellular free calcium was stained for confocal microscopy. The ABR threshold shift after noise exposure was higher in group C than D, and showed a quicker and better recovery in group D than C. Stereocilia loss and the disarrangement of outer hair cells were observed, with the greatest changes seen in group C, followed by groups D and B. The most intensive immunohistochemical intracellular expressions of calmodulin, F-actin, and HSP70 were found in group D, followed by groups C, B and A. The highest intensity of the fluorescent intracellular free Ca^{2+} staining in the isolated outer hair cells was observed in group C. The ABR and morphological studies confirmed the protective effect from noise trauma of sound conditioning. The protective mechanism of hair cells during sound conditioning was enforced through the increase of cellular cytoskeleton proteins and through the relieving of intracellular calcium overloading caused by the traumatic noise.

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Key words: Sound conditioning, Cochlear hair cell, Noise-induced hearing loss

Noise induced hearing loss (NIHL), its control and prevention are major public health issues in both developing and even developed countries. An interesting phenomenon, termed sound conditioning or cochlea toughening, was reported in individuals who experienced repeated low level noise exposure, who developed resistance to some extent to high level acoustic trauma^{1,2}.

Sound conditioning possesses great potential in the reduction and prevention of NIHL and has drawn extensive interest from investigators and clinicians. However, the mechanisms of the protective effect of sound conditioning are still not fully understood. It is generally accepted that an early prominent pathology resulting from NIHL is the impairment of cochlear hair cells^{3–5}. Since cytoskeleton proteins, such as F-actin, calmodulin and Heat Shock Protein 70 (HSP70) play essential roles in the function of cochlear hair cells, we aimed to study the changes of these cytoskeleton structural proteins and the calcium homeostasis within the cochlear hair cells of guinea pigs during conditioning noise exposure.

Materials and Methods

Animal use and experimental grouping

The animal use protocol was approved by the Animal and Human Use in Research Committee of The Institute of Health and Environmental Medicine, Tianjin, PR China. Forty healthy adult guinea pigs (250–350g) (Experimental Animal Center, The Institute of Health and Environmental Medicine, Tianjin, PR China) were used in the study, half of which were male. Preyer's reflex test was normal in all the animals. The animals were kept in a quiet room with constant temperature ($25 \pm 2^\circ\text{C}$) and humidity (50–70%) with a 12-h light/dark cycle (light on from 07:00–18:00 h). The noise level of the quiet room in which the animals were kept was close to 40 dB.

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The animals were randomly divided into four groups:

Group A: controls with no noise exposure, n=10;

Group B: conditioning noise exposure (4 kHz octave band noise, 92 dB for 4 h/day for 7 days), n=10;

Group C: high level noise exposure (4 kHz octave band noise, 110 dB for 1 h), n=10;

Group D: conditioning noise exposure as Group B, followed by a high level noise exposure as in Group C after 2 days rest, n=10.

Set ups for noise exposure

Narrow octave band noise with a center frequency of 4 kHz was generated by using a noise generator (Bruel and Kjaer Instruments, 1027), amplified with a power amplifier, and delivered to a loudspeaker. All exposures were carried out in a reverberation chamber, in a ten wire-mesh cage placed in the center of the sound field, with one animal per cage. The loudspeaker was suspended directly above the cage. Noise levels were measured with a sound level meter (Bruel and Kjaer Instruments, 2209), using a linear weighting at the level of the animals' ears. The noise level variation was less than 1 dB within the space available to the animal. The background noise level in the chamber was below 40 dB sound pressure level (SPL).

Auditory brainstem response (ABR) recording

The ABR was tested and the threshold was recorded before and after noise exposure. For the animals in groups C and D receiving high-level noise exposure, ABR recording was carried out every day in the week after high-level noise exposure.

The guinea pigs were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). A stainless-steel recording electrode was placed subcutaneously on the vertex with the reference electrode on the mastoid and the ground in the mouth. The stimulus signal was generated through Intelligent Hearing Systems (Spirit, USA) controlled by computer and delivered by an earphone. The evoked response was averaged for 1,024 sweeps at each intensity level. Stimuli were presented at a repetition rate of 11.1/s. The threshold was defined as the lowest intensity at which a visible ABR wave was seen in two averaged runs. Threshold shift after noise exposure was calculated with the pre-noise baseline.

Cochlear sensory epithelia surface preparation for microscopic observation

The animals were decapitated under anesthesia after the last ABR recording. The cochleae were immediately removed. The left side was designated for cochlear sensory epithelia surface microscopic investigation and the right side for immunocytochemical study. To ensure efficient fixative perfusion, the round window and the

cochlear apex were opened after the stapes was removed.

The cochleae were prepared with the following procedures for surface microscopic examination. The cochleae were perfused then immersed in 0.5% AgNO₃ solution for 15 min at room temperature, followed by fixation with 10% formalin for 24 h. The specimens were exposed to sunlight for about 2 h until the color of the cochlear surface turned dark brown. The bony capsule was removed and the spiral ligament and stria vascularis were separated under a dissecting microscope. Each turn of the organ of Corti was detached from the bony modiolus, the sensory epithelium was trimmed and then mounted in glycerine on glass slides. The sensory epithelia surface structures were carefully examined for the epithelia cellular arrangement and any missing cells or stereocilia under the biological microscope (Olympus, BH-2) at a magnification of 400 ×. The missing hair cells, stereocilia and disarrangement were counted and photographed along the entire basilar membrane. The percentages of missing inner hair cells and outer hair cells in each turn of the cochlea were calculated and compared among the groups.

Cochlear sectioning and immunohistochemical preparations for labeling of calmodulin, F-actin and HSP70

The right cochleae were rapidly perfused via small holes in the apex and the round window with 10% formalin and immersed in the solution for 24 h. After rinsing in 0.1 M sodium phosphate buffer, pH 7.4, the cochleae were decalcified in 10% ethylenediamine tetraacetic acid (EDTA) solution for 14 days. The specimens were first embedded with 10% and then 25% gelatin, for 24 h at 37°C. The cochleae were sectioned by using a cryostat (Optical, USA) and the sections were collected on nickel grids coated with the adhesive.

The sections were labeled in a moist chamber by immersing them in drops of solution in the following sequence (the number of repeated steps is given in square brackets): 0.3% H₂O₂ for 5 min; 0.05 M Tris-buffered saline, pH 7.4 (TBS) containing 0.1% Triton X-100 for 5 min [× 3]; 10% horse blocking serum in TBS for 10 min; mouse monoclonal antibody to calmodulin (Sigma) at 1/100 dilution in TBS containing 1% bovine serum albumin (BSA) for 2 h at 37°C and then overnight at 4°C; TBS for 5 min [× 3]; anti-mouse Bio-IgG diluted 1/150 in TBS for 45 min at 37°C; TBS for 5 min [× 3]; HRP-SA diluted 1/200 in TBS for 45 min at 37°C; TBS for 5 min [× 3]; DAB staining for 10 min; distilled water; hematoxylin staining for 30 s; and distilled water rinsing. The sections were covered with resin before microscopic observations.

Cell preparation for calcium imaging with fluo-3

The basilar membrane of the cochlea was dissected in

Fig. 1A. ABR threshold of pre and post-noise exposure for each conditioning day.

Fig. 1B. ABR threshold shift induced by high level noise in each day post-noise for group C and group D. Group C, high level noise exposure; Group D, conditioning noise exposure followed by a high level noise exposure. ** $p < 0.01$.

a Petri dish filled with Hank's balanced salt solution (HBSS) and incubated in HBSS with 0.5 mg/ml papain (HBSS) for 10 min at 37°C, followed by rinsing with HBSS. The dishes were coated with poly-L-lysine.

The calcium-sensitive dye, fluo-3/AM (Molecular Probes, USA), was dissolved in dimethyl sulfoxide, diluted in 1 mM and stored at -20°C. The isolated cells were loaded with 10 μ M fluo-3/AM diluted in HBSS for 45 min at 37°C, and rinsed with fresh HBSS. Ca^{2+} in hair cells was examined with laser scanning confocal microscopy (LSCM; Bio-Rad, USA). The fluorescence value of the whole cell was averaged by using software provided by LSCM.

Statistical analysis

Data are expressed as mean $\pm$ S.D. SPSS13.0 (SPSS Inc.) software was used for statistical analysis. Unless specified otherwise, data were analysed by one or two factor ANOVA (general linear model) and two-tailed t-tests. A value of $p < 0.05$ was considered to be statistically significant.

Results

ABR threshold shifts after noise exposure

ABR threshold shifts decreased from 17.6 dB to 3.6 dB during the conditioning noise days, indicating that the conditioning noise was effective (Fig. 1A). A significant difference was observed in ABR thresholds between groups C and D after the high level noise exposure. ABR thresholds in group C and D were 41.4 dB and 28.4 dB, respectively. In this study, noise conditioning protected the cochlea from acoustic trauma by shifting the ABR threshold by 13 dB. In the week after noise exposure, the ABR threshold recovered more

quickly in group D than in group C, and the threshold shifts recovered to 11.0 dB in group C and 1.2 dB in group D, but still did not return to the baseline (Fig. 1B).

Cellular morphological changes of the cochlea

Missing outer hair cell (OHC) stereocilia was observed mostly in outer hair cells but also in a few inner hair cells (Fig. 2). Group C showed the highest percentage of missing OHC stereocilia, followed by group D and group B (Fig. 3), and it was sporadically seen in the controls.

The disarrangement of OHC stereociliary bundles was another morphological finding. It was more obvious in the animals with high level noise exposure (Fig. 2).

Immunohistochemical expression of Calmodulin, F-actin and HSP70 in hair cells

In the control group, calmodulin (CaM), F-actin and HSP70 were immunohistochemically stained uniformly throughout the cytoplasm in the hair cells of the Corti organ. The expression of the three proteins increased after the noise exposure compared to the controls, with the group D showing the highest expressions (Fig. 4).

Ca^{2+} fluorescence staining of isolated outer hair cells

The fluorescent probe fluo-3 stained isolated outer hair cells for Ca^{2+} , uniformly throughout the cytoplasm and higher in the central area of the nucleus. Increased Ca^{2+} staining was seen in cells from the guinea pigs in group C which received noise exposure with the highest intensity (Fig. 5).

Discussion

Sound conditioning was reported as repeatedly conditioned noise exposure which produced certain

Fig. 2. A through D represents the experimental groups A through D as: A, control; B, conditioning noise exposure group; C, high level noise exposure group; and D, conditioning noise exposure followed by a high level noise exposure group. The arrows indicate sporadic OHC stereocilia missing and disarrayed OHC stereocilia.

Fig. 3. The percentage of missing OHC stereocilia in each turn of the basilar membrane. The experimental groups were as described in Fig. 2. * $p < 0.05$, ** $p < 0.01$, # $p < 0.05$, ## $p < 0.01$.

protective effects to later exposure to traumatic noise. It has been demonstrated in the cat, guinea pig⁶⁾, rabbit⁷⁾, chinchilla^{8,9)} and human¹⁰⁾. Noise of 85–100 dB SPL has been proved to be the best intensity for inducing the effect of sound conditioning, and the effect is in proportion to noise intensity in this narrow range^{11–13)}. However, higher intensity means more serious damage to the auditory

system. So we should choose conditioning noise intensity which is as high as possible but avoids hearing damage. At present, 0.5 kHz or 4 kHz octave band noise is often used in sound conditioning studies. In this study, we chose 4 KHz 92 dB and 4 KHz 110 dB as the conditioning noise and the high level noise, respectively, according to previous studies and our experimental experience.

Fig. 4. The expression of CaM, HSP70 and F-actin in the Corti organ of the cochleae of each group. The experimental groups were as described in Fig. 2. MOD: mean optical density. * $p < 0.05$, ** $p < 0.01$, ## $p < 0.01$.

Fig. 5. Fluorescence intensity indicating intracellular free calcium concentration for each group ($\bar{x} \pm s$, $n=50$). The experimental groups were as described in Fig. 2. * $p < 0.05$, ** $p < 0.01$, ## $p < 0.01$.

Three major hypotheses regarding the mechanisms of sound conditioning have been postulated. (1) Acoustic reflection resulting from middle ear muscle strengthening through noise exposure to attenuated low frequency sound, which reduces the temporary threshold shift and permanent threshold shifts¹⁴. (2) The activation of efferent nerve fibers reduces temporary threshold shifts in outer hair cells¹⁵. (3) Increasing cochlear metabolic energy reduces threshold shifts preventing NIHL. In the pathological process of NIHL, the hair cells are the first to be injured after traumatic noise exposure. Therefore,

it is logical to postulate that changes in hair cells, especially the cytoskeleton structures, tend to be the most early and essential mechanisms of sound conditioning. However, this has not been addressed in the literature.

In this study, the ABR threshold shifts showed that the sound conditioning protected the ABR threshold by 13 dB from NIHL. This confirmed both the effects of the noise exposure and sound conditioning, and also demonstrated an effective animal model for future experiments.

The ABR threshold shift recovery after traumatic noise

exposure was quicker in the sound conditioned animals than in the traumatic noise group. This further suggests the protective effect of sound conditioning.

The most prominent and correlative morphological changes in the NIHL are damage and loss of the stereocilia, change of the F-actin level, and reduced cellular energy metabolism^{8, 16–18}. We confirmed the loss of stereocilia of hair cells induced by noise exposure in this study and the severity of the morphological changes correlated with ABR threshold shifts. Our data also showed that the sound conditioning protected hair cells from stereocilia loss following traumatic noise exposure. The uncoupling or immediate damage of the stereocilia might provide a means of protection against noise trauma¹⁹. These damaged hair cells may conserve the potential for possible hearing preservation and recovery. The changes underneath the damages, especially the intracellular skeleton structures, proteins and calcium ion homeostasis, are important information for understanding the pathology of NIHL and also the changes in hair cells damaged by sound conditioning.

It is well known that exposure to traumatic noise produces variable types of cellular structural damage within the cochlea^{1, 17, 20}. The cochlear sensory cells, particularly outer hair cells, responsible for the cochlea mechanoelectrical transduction are most vulnerable. The automatic motility of outer hair cells endows the cochlea with the function of feedback regulation, modulating the hair bundle deflection through the electromechanical transduction. As a main component of hair cell skeleton, F-actin provides a substantial base for motility of outer hair cells, and is thought to be concerned with the pathological changes of hair cells at the advanced stage^{8, 20, 21}. CaM is a major intracellular calcium receptor found in many different cell types, which maintains intracellular calcium homeostasis, and as a result protects cytoskeleton from degradation. Interacting with F-actin, CaM participates in the automatic movement of outer hair cells and regulates hearing acuity based on the calcium-sensitive contraction process^{22, 23}. As an environmental stressor, noise can cause the increase of the protective stress protein, HSP70.

In this study, we found CaM, F-actin and HSP70 increased after noise exposure and their highest levels were found in the animals with sound conditioning followed by the traumatic noise. This result suggests that the up-regulation of the CaM, F-actin and HSP70 may be a protective response of hair cells to noise stimulus, and the sound conditioning reinforced this response. Theoretically, increases of F-actin make the hair cells structurally stronger to mechanical vibration from sound stimulus. A possible mechanism for the increase of HSP70 is that sound stimulus promoted protein biosynthesis and damaged cell renovation.

Because of the availability of isolated hair cells, only

the outside third array of outer hair cells were examined for intracellular free Ca^{2+} . Significantly increased intracellular free Ca^{2+} was found in the animals with traumatic noise exposure, indicating that intracellular calcium overloading was one of the most important mechanisms in NIHL. We postulate that the sound conditioning relieved intracellular calcium overloading, leading to lower levels of intracellular calcium in the animals with sound conditioning prior to traumatic noise. The Ca^{2+} -CaM bound plasma membrane calcium adenosine triphosphatase (PMCA), thus facilitated Ca^{2+} active transport through PMCA.

In conclusion, the results of the ABR and morphological studies presented in this paper showed confirmation of the protective effect of sound conditioning from traumatic noise. Sound conditioning induced the protective mechanism of the hair cells through increases in cytoskeleton proteins and through relieving intracellular calcium overloading caused by traumatic noise.

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