The Protective Effect of Conditioning on Noise-Induced Hearing Loss Is Frequency-Dependent

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Abstract- We compared the extent of temporary threshold shift (TTS) and hair cell loss following high level 4 kHz noise exposure with those preconditioned with moderate level 1 and 4 kHz octave band noise. Fifteen Male albino guinea pigs (300- 350 g in weight) were randomly allocated into three groups: those exposed to 4 kHz octave band noise at 102 dB SPL (group 1, n=5); those conditioned with 1 kHz octave band noise at 85 dB SPL, 6 hours per day for 5 days, then exposed to noise (group 2, n=5); those conditioned with 4 kHz octave band noise at 85 dB SPL, then exposed to noise (group 3, n=5). An hour and one week after noise exposure, threshold shifts were evaluated by auditory-evoked brainstem response (ABR) and then animals were euthanized for histological evaluation. We found that TTS and cochlear damage caused by noise exposure were significantly reduced by 1 kHz and 4 kHz conditioning ($P<0.001$). We also showed that 4 kHz protocol attenuates noise- induced TTS but no significant TTS reduction occurred by 1 kHz conditioning. Both protocol protected noise-induced cochlear damage. We concluded that lower tone conditioning could not protect against higher tone temporary noise-induced hearing loss, thus conditioning is a local acting and frequency-dependent phenomenon.

Introduction

Noise-induced hearing loss (NIHL) is the most common cause of occupational hearing loss. Therefore, the scientists are looking for ways to control noise level at working places and encourage labors to wear hearing protective devices, but they are not yet fully succeed. In the other hand, NIHL is not limited to working places, as people are often exposed to high level of noise on their routine life. Today, many researchers are involved in finding various methods of increasing the resistance against noise- induced hearing damage. Pharmacological intervention is being extensively studying, but few medicines are yet clinically available.

One of the methods known to increase the resistance of cochlea is sound conditioning. It is well studied that if we expose the cochlea to low level noise, it will be protected against subsequent noise trauma (1). It has been shown protective in mammals and human (2,3).

However, its mechanism of action is still controversial. The previous studies speculated that conditioning may involve innate repair system such as antioxidants and free radical scavengers, heat shock proteins, and neurotrophic factors. It is also been shown that glucocorticoids, and inhibition of apoptosis play an important role (4). The question raised is whether the mechanism of protection by conditioning acts locally or systemic. In our knowledge, most of previous studies have used the same frequencies of noise in conditioning and trauma paradigm. It means they exposed subjects to traumatic level of noise at the same frequencies as conditioning. We aimed to evaluate the effect of conditioning the cochlea by a lower tone noise against subsequent higher tone noise trauma. Therefore, we studied the animals conditioned by 1 and 4 kHz low level octave band noise, and then exposed them to 4 kHz high level octave band noise. We compared the effects of 1 kHz- and 4 kHz- conditioning on 4 kHz noise trauma by means of electrophysiological and

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histological evaluation. The results of our study speculated that conditioning has a local mechanism.

Materials and Methods

Animals

This study was conducted on 15 albinos, male guinea pigs (Pasteur's Institute, Iran). The animals (300- 350 g) were considered normal by Preyer's reflex. They were randomly assigned to three groups:

Group 1: Animals exposed to 4 kHz octave band noise without preconditioning (5 ears).

Group 2: Animals conditioned with 1 kHz octave band noise, then exposed to 4 kHz traumatic noise one day later (5 ears).

Group 3: Animals conditioned with 4 kHz octave band noise, and then exposed to 4 kHz octave band noise one day later (5 ears).

This study was reviewed by the Committee for Ethics in Animal Experiments of Tehran University of Medical Sciences.

Experimental protocol

Guinea pigs were housed in a quiet and ventilated room for 2-3 days after arrival. Subjects in group 1 were exposed to traumatic noise (day 9), then examined by auditory-evoked brainstem response (ABR) an hour later. Subjects in groups 2 and 3 exposed to conditioning sound (day 4- 8), then exposed to traumatic noise (day 9) and their temporary threshold shifts (TTS) were evaluated an hour later by ABR. On day 16, all subjects were evaluated by ABR, and then sacrificed for hair cell count. The noise considered to induce TTS was 4 kHz octave band noise at 102 dB SPL for 3 hours. For conditioning, we considered two protocols of 1 kHz and 4 kHz octave band noise at 85 dB SPL, 6 hours per day for 5 days. In a pilot study, we examined two animals right after each conditioning protocol to exclude any ABR threshold shift caused by the sound conditioning.

Noise exposure

All exposures were carried out in a lighted, ventilated and sound-deadened chamber, with two wire-mesh cages located at the corners. One animal per cage was exposed and the guinea pigs were allowed to move freely with access to water and food during exposure. The sound chamber was fitted with speakers centrally hanged from the roof and driven by a noise generator and power amplifier. A 0.5 inch Bruel and Kjaer condenser microphone and Fast Fourier Transform analyzer were used to calibrate and measure sound

levels at corners within the sound chamber to ensure stimulus uniformity of ± 1 dB within the exposure area.

ABR measurement

Hearing thresholds in the right ears of all animals were evaluated. The guinea pigs were anesthetized with a mixture of xylazine (4 mg/ kg, i.m.) and ketamine (40 mg/ kg, i.m.). To measure ABR threshold (ECLIPS EP25, Interacoustic, Denmark), needle electrodes were placed subcutaneously below the test ear (inverting), and at the vertex (non- inverting). A ground electrode was positioned at the back of animal. The sound stimulus consisted of a 15 ms tone burst, with a rise-fall time of 1 ms at frequencies of 1 and 4 kHz. The intensity was varied in 5 dB steps. Hearing thresholds were defined by visual interpolation between the lowest intensity producing a definite, repeatable response and an intensity 5 dB less, at which no ABR response was elicited. Threshold shifts were determined by subtracting the hearing threshold after noise exposure at each frequency from values obtained before exposure.

Histological examination

The right temporal bones were immediately excised after decapitation under deep anesthesia. As mentioned elsewhere (5), under a dissecting microscope, the perilymphatic spaces were perfused for 1 h with 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4, then washed in buffer. For specific F-actin staining whole mounts of the organ of Corti were stained with rhodamine phalloidin for 60 min after being made permeable by 0.3% Triton X-100 treatment for 5 min (6). The tectorial membrane, Reissner's membrane, osseous spiral lamina and cochlear nerve were removed under a dissecting microscope, and the individual turns of the organ of Corti mounted on glass slides.

Specimens were observed under a fluorescence microscope, from the area next to the apex to the base in all the animals. Hair cells that showed an identifiable cell body and cuticular plate were considered to be present. Distinctive scar formation produced by convergence of adjacent phalangeal processes was regarded to show a missing hair cell (7). The cells in the area compatible with 4 kHz frequency map, according to our previous study (8), approximately in lower 2nd and upper basal turn of the cochlea (9- 15/5 mm from the apex) were counted.

Statistical analysis

Sigma Stat^{TM} statistical software was used. Thresholds in each group were analyzed statistically by

paired t- test. ABR threshold shifts at each frequency, as well as the percentages of missing OHCs, were compared for groups by a one-way analysis of variance (ANOVA). Significant differences found were compared with each other by the Tukey test. The 0.05 level of probability was the criterion for statistical significance.

Results

Electrophysiological findings
The hearing thresholds before noise exposure were essentially equivalent in all the ears. There were no significantly differences at any frequency across groups. The results of ABR thresholds before and an hour after noise exposure in each group showed in figure 1.

In control group using 1 kHz- and 4 kHz-tone bursts stimuli, auditory thresholds an hour post- noise exposure were significantly higher when compared with pre-noise and one week post- noise exposure ($P<0.001$), however, there were no statistically significant differences between pre-noise and one week post-noise exposure data. This means that ABR thresholds showed significant TTS which almost recovered to baseline level. We also compared the average of 1 kHz- and 4 kHz-tone burst ABR thresholds in 3 different time scales. ABR thresholds had no meaningful differences between 1 kHz- and 4 kHz-tone bursts measured pre-noise exposure. The mean of thresholds using 4 kHz- were significantly higher than 1 kHz- tone bursts ($P<0.01$) an hour post- noise exposure but had no

significant differences one week later. These results showed the most temporary threshold shift at 4 kHz-tone burst.

In the 1 kHz- conditioned group using 1 kHz- tone burst stimulus, although there were threshold shifts an hour post noise exposure, it was not statistically important when compared with pre- noise exposure, but significantly higher when compared with one week post-noise exposure ($P<0.01$). However by 4 kHz stimulus, auditory thresholds an hour post- noise exposure were significantly higher compared with pre-noise and one week post- noise exposure ($P<0.001$), There were no statistically significant differences between pre-noise and one week post-noise exposure data using both stimuli. These mean that ABR thresholds showed significant TTS which almost recovered to baseline level when recorded by 4 kHz- tone burst, but no significant TTS observed after 1 kHz- conditioning when measured by 1 kHz- tone burst. We also compared the average of 1 kHz- and 4 kHz-tone burst ABR thresholds in 3 different time scales. ABR thresholds had meaningful differences between 1 kHz- and 4 kHz-tone bursts measured pre-noise exposure, showing lower threshold by 1 kHz- stimulus ($P<0.05$). The mean of thresholds had no significant differences an hour post-noise exposure but 4 kHz- had significantly higher threshold than 1 kHz-tone bursts one week later ($P<0.01$). These results showed that 1 kHz- conditioning attenuated temporary threshold shift using 1 kHz-tone burst, but its protective effect was not significant when ABR recorded by 4 kHz- stimulus.
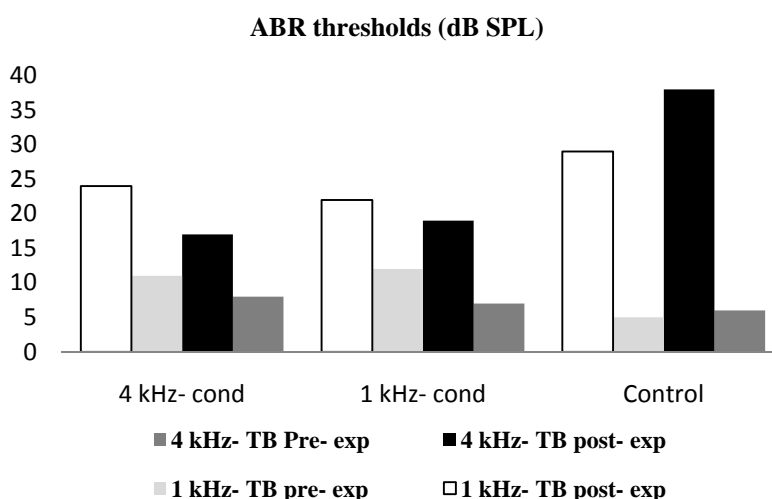
ABR thresholds (dB SPL)

Figure 1. ABR thresholds an hour after (post- exp) and before (pre- exp) noise exposure in (4 kHz and 1 kHz) conditioned and control groups. Bars in each group display thresholds using 1kHz- (1kHz- TB) and 4kHz- (4kHz- TB) stimuli post- and pre-exposure, respectively. ABR thresholds recovered to baseline level one week later (data not shown).
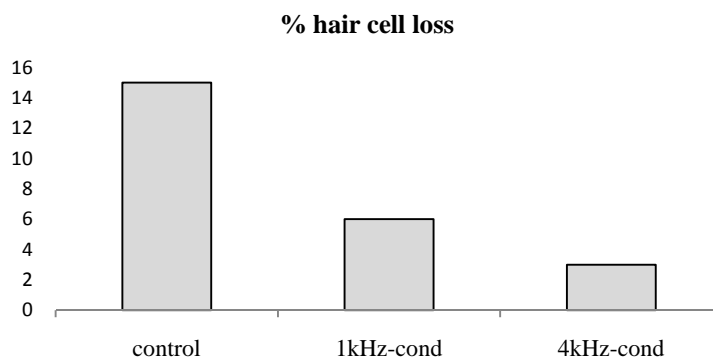
% hair cell loss

Figure 2. The percentages of hair cell loss (row 1) in control and conditioned groups one week after noise exposure.

In the 4 kHz- conditioned group using 1 kHz- and 4 kHz-tone bursts stimuli, auditory thresholds an hour post- noise exposure were significantly higher when compared with pre- noise and one week post- noise exposure ($P<0.01$), however, there were no statistically significant differences between pre- noise and one week post- noise exposure data. This means that ABR thresholds showed significant TTS which almost recovered to baseline level. We also compared the average of 1 kHz- and 4 kHz-tone burst ABR thresholds in 3 different time scales. ABR thresholds had no meaningful differences between 1 kHz- and 4 kHz-tone bursts measured pre-noise exposure. The mean of thresholds at 4 kHz- were lower and statistically meaningful compared to 1 kHz-tone bursts ($P<0.05$) an hour post-noise exposure and had no significant differences one week later. These results showed that 4 kHz- conditioning attenuated temporary threshold shift using 4 kHz-tone burst.

Histological findings

By surface preparation method used in this study and under fluorescent microscope, we observed inner hair cells, outer pillar cells, outer hair cells row 1, Deiters' cells row 1, outer hair cells row 2, Deiters' cells row 2, outer hair cells row 3, Deiters' cells row 3.

In control group, we evaluated hair cell loss after exposure to traumatic noise without preconditioning. We observed the whole body of cochlea from the base to the apex and found an area of maximum damage around the second turn of the cochlea (approximately 11 mm from the apex). In this region, there was some outer hair cell loss observed. In the remaining areas of the cochlea, there was some spare hair cell loss, mostly seen in outer hair cell row 1. There were no significant damage to supporting cells and inner hair cells. We found no specific abnormality when observed stereocilia.

In conditioning groups, there was no significant damage to inner and supporting hair cells. Stereocilia had no abnormality ranging from floppy, disarrayed, broken tip links and broken roots to collapsed, fused and elongated stereocilia. There was spare hair cell loss, mostly seen in outer hair cell row 1. In comparison to control, cells were well preserved.

In order to compare the effect of different frequency of conditioning on hair cell loss, the average of outer hair cell loss row 1 in the area of 9-14 mm from the apex were counted and compared with each other. Figure 2 showed that hair cell loss in conditioning groups were significantly lower than control group ($P<0.05$) while there was no meaningful difference between two conditioning groups in the amount of hair cell loss.

Discussion

This study aimed to evaluate the effect of sound conditioning against subsequent noise-induced hearing loss and cochlear damage. We preconditioned animals with 1 and 4 kHz octave band of noise at 85 dB SPL, 6 hours/day for 5 days then exposed them to 4 kHz octave band of noise at 102 dB SPL for 3 hours almost 16 hours later. We evaluated tone burst ABRs at 1 and 4 kHz an hour and one week post exposure and then analyzed hair cell loss. Electrophysiological evaluation showed significant ABR threshold shifts, and histological evaluation showed some hair cell loss following exposure to traumatic level of noise. However, threshold shift and cochlear damage were significantly reduced by both 1 kHz and 4 kHz conditioning paradigms. Comparing the results of 1 kHz and 4 kHz conditioning, there were no meaningful differences in threshold shift caused by conditioning with 1 kHz followed by 4 kHz noise trauma; however 4 kHz- conditioning significantly reduced threshold shift subsequent to 4 kHz noise trauma. Our results showed that conditioning protects

against noise- induced TTS and cochlear damage, however, lower tone conditioning is not protective against higher tone noise trauma. Therefore, we concluded that conditioning is a local- acting and frequency- dependent phenomenon.

In this study, exposure to traumatic noise induced significant threshold shift. An octave band of noise has a unified energy at the specific frequency, for example Harding *et al.* (2007) stated that 4 kHz noise has a maximum energy around 3-6 kHz, reducing at least 30 dB SPL in upper and lower frequency area (9). In control group of current study, exposure to 4 kHz octave band of noise caused considerable TTS, which were statistically different when measured by means of 4 kHz- compared to 1 kHz- tone burst stimulus. This result showed higher TTS measured by 4 kHz- tone burst which can be explained by maximal energy level of noise used in the study.

We used 4 kHz OBN at 102 dB SPL to induce cochlear damage. It is known that cochlea has high metabolism and the high level of energy needed for cochlear metabolism is supplied by mitochondria located mainly within stria vascularis. Noise exposure induces excessive reactive oxygen species (ROS) production in mitochondria. When high level of ROS exceeded endogenous antioxidant capacity, it damages lipids, proteins and nucleic acids causing cell death. In a previous study using 4 kHz noise, the most damaged area was somewhere around the end of basal and the beginning of second turn of the cochlea, it was calculated to be approximately 9.0-15.5 mm from the apex (5), it is consistent with the guinea pig cochlear frequency map (10). In current study, we found the most damaged area around 11 mm from the apex which is somewhere around the lower 2nd turn of the cochlea, compatible with the area received the most energy of 4 kHz noise. We found some outer hair cells, showing injury in this region. Different stiffness in the basal and the apex of cochlea results in the impedance gradient in basilar membrane of the organ of Corti. This mechanical characteristic is necessary for cochlear acoustic processing. Any injury to pillar cells, as the main supporting cells, will change this impedance gradient and influence cochlear sound analyzing. On the other hand, outer hair cells are the most sensitive cells in the organ of Corti. Its mobility causes depolarization and frequency tuning and sensitivity amplification. In current study, as expected, the most damaged cells were outer hair cell row 1 while inner hair cells and supporting cells were well preserved. It is known that

noise-induced hearing loss starts with outer hair cell damage. By increasing intensity and duration of exposure, pillar cell and inner hair cell injury can be gradually observed. ABR threshold shifts also returned to basal level one week after noise exposure. Therefore, we speculated that noise used in the control group was suitable to induce TTS.

The pattern and extent of cochlear damage depends on when we evaluate hearing loss and cochlear damage. We had higher TTS at 4 kHz which was explained by histological data. However, there were also some threshold shifts at 1 kHz which is out of the most damaged area. Stereocilia connection to tectorial membrane is critical for mechano-electrical transduction. Any abnormality including floppy, disarrayed, fused, or broken stereocilia (11) on sensory cells may influence potassium influx from mechano-electrical canals which is necessary for cellular depolarization. We could not quantify damage to stereocilia, because of our technical limitation. Considerable TTS at 1 kHz can be explaining by the subtle changes in hair cell which could not be evaluated by surface preparation method used in this study. Some of temporary changes such as cellular swelling and stereocilia's damage also recover a few days after exposure causing less threshold shift.

Conditioning in our study attenuated ABR threshold shift and hair cell loss. There are two different methods to reduce inner ear sensitivity against subsequent intense noise exposure; by a low- level, continuous non-traumatic acoustic stimulus preceding severe traumatic noise (conditioning) and by stimulus producing temporary threshold shift in the first days of exposure (toughening) (2). It is reported that the stimulus not producing any threshold shifts or hair cell damage is the most preventive against subsequent hearing loss and hair cell injury (12). The protocol used in this study was conditioning with 85 dB SPL with almost 16 hours rest between conditioning and noise trauma, enough time for recovering to the baseline level even if any probable TTS was occurred. In addition, in a pilot study, we examined two animals right after each conditioning protocol to exclude any ABR threshold shift caused by the sound conditioning. This means that the conditioning protocol was suitable in this investigation. Thresholds were recovered after conditioning when compared to control. It is hypothesize that conditioning activates multiple endogenous protective mechanisms including antioxidants or ROS scavengers (13), calcium buffering system (14), heat shock proteins (15), glutamate receptors and neurotrophic factors (4). Canlon and

coworkers (1992) reported protection by conditioning in guinea pigs (14). Electrophysiological data showed reduced threshold shifts in conditioning compared to their control group. They confirmed that sound conditioning causes resistance against noise trauma. Zuo and coworkers (2008) also investigated threshold shift and hair cell injury in mice. Electrophysiological and histological data showed that conditioning is protective (16).

To answer the question about whether the mechanism of conditioning acts locally or systemic, we compared the results of two conditioning groups. When we condition the cochlea by 1 kHz OBN, the travelling wave activates cochlea from base to apex approximately from 20 to 1 kHz area. We investigated whether this activation is enough to stimulate endogenous antioxidant system and attenuate damage in its whole activated area. When we condition the cochlea by 1 kHz OBN, significant TTS protection at 4 kHz area was not occurred. However, protection by 4 kHz conditioning attenuated TTS significantly. Therefore, lower- tone conditioning could not protect higher frequency noise trauma, but reduced TTS when ABR measured with 1 kHz tone burst. In conclusion, our study confirmed the protective effect of conditioning and showed that this protection is frequency dependent. Using method of current study, the conditioning revealed a local mechanism.

Acknowledgement

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