

Original Article

Effects of edaravone treatment on noise-induced hearing loss

Yi-Deng Huang^{1,2*}, Long-Jun Wu^{3*}, Si-Wen Xia², Yang Zhuo², Geng-Tian Liang³, Ning Yu¹, Wei-Wei Guo¹, Shi-Ming Yang¹, Dong-Yi Han¹

¹Department of Otolaryngology-Head and Neck Surgery, Otolaryngology Institute of PLA, General Hospital of PLA, Beijing 100853, China; ²Department of Otolaryngology, 118th Hospital of PLA, Wenzhou 325000, Zhejiang, China; ³Department of Otorhinolaryngology, The Third Hospital of Wuhan, Wuhan 430000, Hubei, China. *Equal contributors and co-first authors.

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Abstract: This study investigated the effects of edaravone on the cochlear hair cells of guinea pigs after noise-induced hearing loss and the best time for drug administration. Thirty clean-grade guinea pigs were randomly divided into three groups, and auditory brainstem responses (ABRs) were measured before, instantly after, and on the 3rd and 7th day after noise exposure. On the 8th day after noise exposure, succinate dehydrogenase immunohistochemistry and scanning electron microscopy (SEM) were performed. The ABR threshold of the group that was administered edaravone immediately after the noise exposure was lower compared with that of the saline group ($P < 0.05$). Three days later, the ABR threshold in the group was decreased more. Seven days later, it was similar to the ABR of the normal control group, while the ABR of the saline group was significantly higher than that of the normal control group ($P < 0.05$). Light microscopy revealed that the cytoplasm of the outer hair cells was more intensely stained in the edaravone group compared to the saline group. The cellular morphology, immunohistochemistry, and SEM analyses showed that the edaravone group exhibited significantly less nuclei and cilia deficits than the saline group did. The early intraperitoneal injection of edaravone had beneficial effects on the cochlear hair cells of guinea pigs after noise exposure injury. Edaravone was effective when administered within 1 week of the noise exposure, but it was most effective when it was administered within 3 days of the noise exposure.

Keywords: Edaravone, noise-induced hearing loss, cochlea

Introduction

Noise injury to cochlear hair cells is a common cause of deafness. After noise exposure, reactive oxygen and reactive nitrogen contents, which reflect oxidative stress, are significantly increased in the cochlea, and the oxidative stress results in cochlear damage [1, 2]. Capaccio et al. found that oxidative stress damaged the capillaries of the inner ear tissues and that the cochlear ischemia and hypoxia increased the production of free radicals, thus forming a vicious cycle that resulted in hearing loss [3].

Currently, the main antioxidants that are used clinically are edaravone, glutathione, and ascorbic acid [4]. Edaravone is a new and very effective anti-free radical scavenger that has lipo-

philic groups. Edaravone has been used in a number of neurological applications, and it exhibits significant protective effects on free radical-induced nerve cell damage [5].

In otology, the scavenging effects of edaravone towards free radicals that were locally generated by noise-induced cochlear injury have been confirmed, and these effects inhibit the death of cochlear hair cells and significantly attenuate hearing loss. Gao et al. reported that the increase in free radicals after noise exposure peaked 2-6 h after noise exposure and then continued after [6]. Three days after noise exposure, the free radical levels were still higher than those in the normal control group [6]. Tanaka et al. found that the effects of edaravone were most significant 21 h after noise exposure [7], and Van Campen et al. found that

the free radical contents were still higher than the normal levels 72 h after the noise exposure with a peak 8 h after the noise exposure [8]. Among the findings described above, the durations reported for the increased free radical levels after noise exposure were not consistent. Therefore, they do not provide a clear reference for clinical treatment.

This experiment simulated noise-induced hearing loss in a guinea pig animal model. The animals were intraperitoneally injected with edaravone within 30 min to 1 week after noise exposure in order to explore the most effective time for edaravone treatment on cochlear injuries and provide a theoretical foundation for the clinical treatment of noise-induced hearing loss.

Materials and methods

Animal grouping

Thirty albino guinea pigs (weighing 200-250 g, specific pathogen-free grade; Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) with normal ear reflections were randomly divided into the following three groups: normal control group, saline group, and edaravone group, with 10 in each group. The edaravone group was administered intraperitoneal injections of edaravone solution (8.0 mg/kg; Sinopharm Guorui Pharmaceutical Co., Ltd., Beijing, China) once a day within 30 min to 1 week after the noise exposure, and the saline group was injected with saline solution (5.4 mL/kg) at the same time points. The control group did not have noise exposure or intraperitoneal injections. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of General Hospital of PLA. All of the surgeries on the albino guinea pigs were performed while the pigs were anesthetized with 4.5% chloral hydrate (0.4 mL/100 g, intraperitoneal injection), and all efforts were made to minimize suffering.

Noise exposure

The animals in the saline and edaravone groups were caged individually. When the animals were

awake, the acoustic noise exposure was performed in a sound isolation room, and the noise was generated by a signal generator (TMS32020, Loughborough Sound Images, Ltd., Leicestershire, UK) that was amplified by a power amplifier (NAD 2200, USA) and passed to speakers (JBL 2360, USA). The speakers were placed in a horizontal position 3 cm in front of the animal's head, and a sound level meter (Larson and Davis 825, USA) was used to measure the noise intensity. The sound intensity of the white noise (300 mV) was 120 dB, and it was performed 4 h per day for 4 days. The normal control group was reared in a quiet environment without noise exposure.

Hearing detection

The auditory brainstem response (ABR) threshold was set as the observation indicator. The recording electrode was implanted under the scalp at the middle point of the connecting line of the binauricular front edges so that it contacted the skull. The reference electrode and grounding electrode with 1-k Ω resistances were placed in both earlobes. The stimulus was a wideband short click, with a pulse width of 0.1 ms. The scanning process was 10 ms for 1,024 superimposition times, and the sound stimulus intensity when the most stable III wave disappeared was set as the auditory reaction threshold of the ear. While anesthetized, the binauricular ABR thresholds of each group were tested and recorded four times [before exposure, immediately after exposure (30 min), and on the 3rd and 7th days after noise exposure].

Succinate dehydrogenase (SDH) staining

On the 8th day after noise exposure, 5 guinea pigs in each group were randomly selected and intraperitoneally anesthetized with chloral hydrate (0.4 mL/100 g). Subsequently, the animals were decapitated in order to dissect the binaural cochleae, which were exposed under a dissecting microscope. The top bony shell and the round window film of the cochlea were then pricked in turn, and the base plate of the stapes was then removed. The freshly mixed SDH dye [0.2 mol/L phosphate-buffered saline (PBS), 0.2 mol/L sodium succinate, and 0.1% nitro blue tetrazolium, at a ratio of 1:1:2] was then slowly infused from the top of the cochlea. After these procedures were repeated five times, the cochlea was incubated in 10 mL of

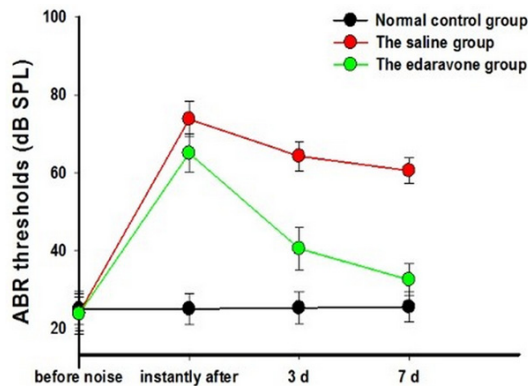


Figure 1. ABR thresholds of the three groups before the noise exposure, the ABR thresholds of the 3 groups had no significant difference, indicating that the intergroup conditions of guinea pigs were of the same ($P>0.05$), the ABR thresholds of the Edaravone group and the saline group after the noise exposure were higher than the normal control group, with the statistically significant difference ($P<0.05$), while that of the edaravone group was lower than the saline group, and the difference was statistically significant ($P<0.05$); 3 d after the noise, the ABR threshold of the edaravone group further reduced, and 7 days later the ABR threshold recovered near to the normal, while that of the saline group was significantly higher than the control group, with the statistically significant difference ($P<0.05$). In addition, the ABR thresholds 7 of the edaravone group, measured immediately after the noise exposure and 7 days after the exposure showed the statistically significant difference ($P<0.05$).

dye at 37°C in the dark for 1 h, which was followed by fixation in 4% formaldehyde solution at room temperature for 4 h. The cochlear basilar membrane was then isolated in 0.01 M PBS and spread in segments onto glass slides for glycerol mounting and optical microscopy examination.

Fluorescence staining of the cochlear hair cells

On the 8th day after noise exposure, 3 guinea pigs in each group were randomly selected and decapitated while they were anesthetized. The cochlea was then quickly removed, and the cochlear duct was perfused with 4% paraformaldehyde solution and fixed overnight at 4°C. The cochlear basilar membrane was then isolated in 0.01 M PBS and washed with 0.01 M PBS three times for 10 min each. The membrane was incubated in phalloidin (P5282; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) for 30 min in order to label the hair cell cilia and

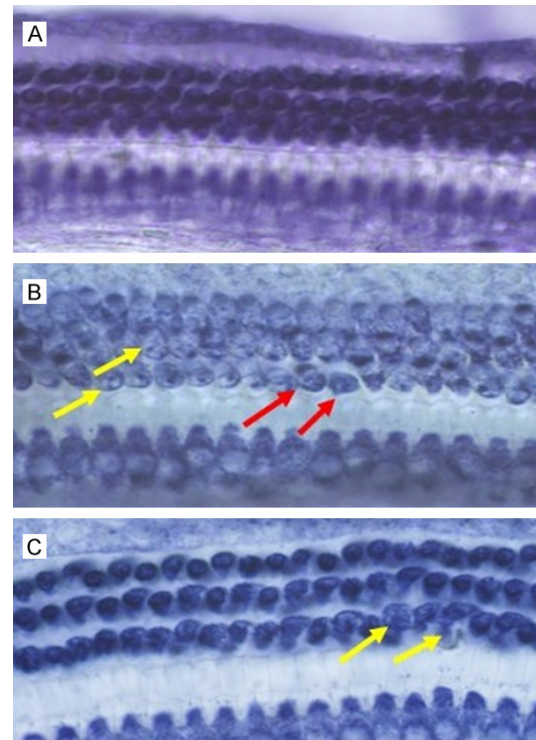


Figure 2. SDH staining of cochlear organ of Corti. A. SDH staining of normal cochlear organ of Corti, the outer hair cells were arranged in neat rows, the cytoplasm was dark blue, showing that the mitochondrial SDH activities were normal. B. The saline group: almost all the outer hair cells were unstained (shown by the yellow arrow), only the cytoplasm of a few scattered outer hair cells was deeply stained (shown by the red arrow); C. The edaravone group, the outer hair cells were arranged in neat rows, with scattered outer cells weakly stained (yellow arrow), and the cellular morphology was irregular (SDH staining $\times 400$).

epidermal plates, and the nuclear DNA fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) was used to restain the nuclei for 10 min, which was followed by glycerol mounting and confocal microscopy examination.

SEM

The 2 remaining guinea pigs in each group were anesthetized, and the cochlea was removed. The cochlea was fixed overnight in a 2.5% glutaraldehyde solution at 4°C. Subsequently, the cochlea was incubated in 10% ethylene diamine tetraacetic acid for full decalcification, and osmic acid was used for fixation. The cochlea was visualized under the microscope in order to remove the bony labyrinth, membranous labyrinth, vestibular membrane, and covering film, and electric conduction staining and isoamyl

Table 1. Changes of ABR thresholds in different stages in the three groups (dB, $\bar{x} \pm S$)

Group	Before noise exposure (n=20)	Immediately after the noise exposure (n=20)	3 days after the noise exposure (n=20)	7 days after the noise exposure (n=20)
A (normal control group)	20.1 \pm 2.1	21.4 \pm 1.3	20.5 \pm 1.7	19.9 \pm 1.9
B (saline group)	21.8 \pm 2.7	73.9 \pm 2.2	65.4 \pm 3.4	60.5 \pm 3.1
C (edaravone group)	19.9 \pm 1.9	65.5 \pm 3.1	39.1 \pm 5.1	32.1 \pm 4.1

acetate ester replacement were then performed. At 4°C, 50%, 70%, 90%, and 100% alcohols were used for gradual dehydration, which was followed by critical point drying (HCP-2 critical point drying apparatus; Hitachi, Ltd., Tokyo, Japan), gilding (E-102 vacuum ion plating apparatus), and SEM observation (Hitachi S-4800; Hitachi, Ltd.).

Statistical analysis

SPSS 13.0 (IBM Corporation, Armonk, NY, USA) was used for the statistical analysis. The figures and charts were created with Sigma Plot10.0 software (Systat Software, Inc., San Jose, CA, USA) for the analysis. Tests of the homogeneity of the variance were performed first in each group. If the homogeneity of variance was met, the intergroup mean values were then compared with a univariate analysis of variance (ANOVA) and *t*-test; if not, a non-parametric test was performed. The rates were tested with the Kruskal-Wallis rank sum test. If the difference was significant, the Nemenyi test was used to compare pairs of samples. *P* values less than 0.05 were considered statistically significant.

Results

Changes in the ABR threshold

Before noise exposure, the ABR thresholds in the guinea pigs in the 3 groups were in the normal range. The ABR thresholds in the edaravone group and saline group were examined after the last intraperitoneal injection was completed, and they were increased. Three days after the noise exposure, the ABR thresholds in the edaravone group and saline group were partially recovered. After 7 days, the ABR threshold in the edaravone group was normalized, while the ABR in the saline group was still increased (**Figure 1**). These findings indicated that the first week after noise exposure was an important stage in ABR threshold recovery, and

the 3 days after noise exposure was the key period for hearing protection.

SDH staining

With visualization under an ordinary optical microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan), the mitochondrial SDH staining area and staining intensity of the hair cells of the organ of Corti were examined in the cochlear basal turn in the 3 groups. The same parts of the organ of Corti in the cochlear basal turn were observed (**Figure 2**).

SEM results

The hair cells of the acoustic papilla in the guinea pigs in the 3 groups were examined with SEM (**Table 1**). The hair cell cilia in the normal control group were arranged in an orderly pattern with a clear structure. After noise exposure, the hair cell cilia were missing, disordered, or dislodged in the edaravone group and saline group. In the saline group, the sporadic absence of lateral ciliated cells predominated. Compared to the saline control group, there were significantly less scattered and missing hair cells in the edaravone group (**Figure 3**).

Morphological changes in the cochlear hair cells

The cilia of normal hair cells exhibit red fluorescence when they are labeled with phalloidin, and the outline of their surfaces are clear. After the labeling of their nuclei with the nuclear-specific fluorescent dye DAPI, a clear blue nuclear structure was observed. Under normal circumstances, the cochlear hair cells were arranged neatly and orderly without missing or damaged cells (**Figure 4A**). Seven days after the noise exposure, the edaravone group and the saline group exhibited sporadic deletion of the outer hair cells at the distal end of the cochlear basement turn and at the beginning of the end of the second turn (**Figure 4B, 4C**), while the severity of deletion in the edaravone group was signifi-

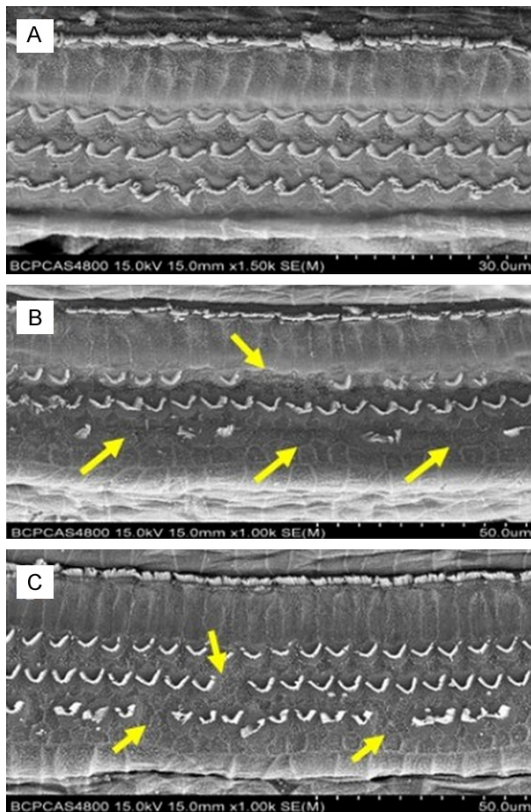


Figure 3. SEM of cochlear organ of Corti. A. The normal control group, the outer and inner hair cells were arranged in neat rows, the cilia of outer hair cells exhibited the V-shaped arrangement, while those of inner hair cells showed line-shaped arrangement; B. The saline group, the cilia of outer hair cells of organ of Corti in the cochlear basal turn exhibited the flaky missing (shown by the yellow arrow), while those of inner hair cells were relatively complete. C. The edaravone group, the outer hair cells were neatly arranged, exhibiting the scattered deletion of cellular cilia, while significantly reduced than the saline group.

cantly reduced compared to that in the saline group.

Discussion

Noise is a sound that involves erratic changes in frequency and intensity. Noise-induced hearing loss, which is one of the most serious occupational hazards worldwide [9], is thought to mainly result from mechanical and metabolic damages, such as ischemia and hypoxia, which both produce large amounts of reactive oxygen species (ROS) inside the cochlear hair cells. In addition, the overload of intracellular Ca^{2+} promotes the generation of more free oxygen radicals, which results in structural dysfunction of

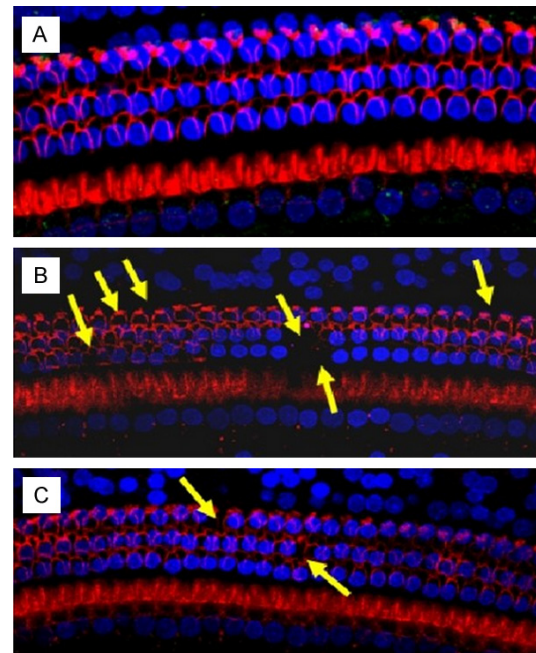


Figure 4. Nucleus and cilia staining of cochlear organ of Corti. A. The normal control group, the nucleus of inner and outer hair cells in the basal turn organ of Corti were arranged neatly, with the epidermal plate stained clearly; B. The saline group, the nuclei of outer hair cells could be seen missing (shown by the yellow double arrows), with the missed outer hair cells (shown by the yellow single arrow); C. The edaravone group, the outer cells were arranged neatly, while with the scattered cellular missing.

the cochlea and hair cells, which eventually leads to hearing loss [10]. In this study, the ABR thresholds of the guinea pigs in the 3 groups before the noise exposure, right after the noise exposure, and 3 and 7 days after the noise exposure were measured in order to determine the effects of edaravone on noise-induced hearing loss. The results showed that there was no significant difference in the ABR thresholds among the 3 groups, but the saline group and edaravone group exhibited increased ABR after noise exposure compared to the control group. The noise was more than 40 dB, which indicated that the noise damaged the cochlear outer hair cells of the guinea pig in only 3 days, but the ABR threshold in the edaravone group was significantly lower than that in the saline group. We therefore inferred that edaravone had protective effects on the cochlea. The ABR threshold in the edaravone group was decreased compared with that in the saline group, and it became almost normal 7 days after noise exposure.

Takemoto et al. found that edaravone had protective effects on cochlear damage that was induced by high-intensity noise (130 dB) [11]. In addition, Maekawa et al. showed that edaravone prevented cochlear damage from acute barotrauma in guinea pigs [12]. Edaravone was protective in the cochlea of rats that were treated with bramycin within 7 days [13]. These results confirmed that edaravone has free radical scavenging effects on cochlear hair cells, and these effects were obvious within one week.

The mechanisms underlying the protective effects of edaravone on the cochlea after noise exposure may be as follows. Within a short period after noise exposure, a large number of free radicals appear in the mitochondria in cochlear cells. The free radicals damage the normal redox equilibrium state of the cochlea, which results in mitochondrial dysfunction and cell disintegration and death [14]. Edaravone exists in anion form in the body, and it directly clears the free radicals around the mitochondria in order to protect the cells. Edaravone transfers an electron to the free radical, thus generating an edaravone group that interrupts the lipid peroxidation chain. Edaravone reduces the oxidative damage of mitochondrial DNA by free radicals, protects the normal functions of the respiratory chain, and improves the mitochondrial energy supply and antioxidant effects, thus resulting in protection of the cells. Edaravone inhibits the release of mitochondrial cytochrome C, thus blocking the JNK signaling pathway and protecting the nerve cells [15]. Specifically, edaravone blocked the shifting of BAX from the cytoplasm to the mitochondria, thereby blocking the release of cytochrome C from the mitochondria to the cytoplasm. Yoshida et al. found that edaravone downregulated the expression of mRNA in liver cells, upregulated the expression of BCL-2 protein, and stabilized the mRNA expression in liver cells to inhibit cellular denaturation and degeneration [16]. Recent studies have shown that edaravone directly scavenges free radicals and significantly reduces the serum levels of inflammatory cytokines [17-19]. These results suggested that edaravone ameliorates cochlear metabolic disorder, scavenges free radicals, reduces late-phase threshold shifting extents, and reduces the permanent threshold shifting in guinea pigs after noise exposure.

In this experiment, the intensity of SDH staining was used to examine mitochondrial activities. SDH, which is a binding enzyme that is located inside the mitochondrial membrane, acts as an indicator of mitochondrial functions. It belongs to the flavin enzyme family and is one of the pivots that connects oxidative phosphorylation and electron transfer. SDH can provide electrons for the respiratory chains that satisfy the oxygen requirements and energy generation of eukaryotic cellular mitochondria and various prokaryotic cells. It is closely related to the formation of ROS. After noise exposure, many intracellular ROS seriously damage mitochondria. Thus, the SDH staining inside the hair cells will be weakened, or it will not be seen. This is consistent with Hu et al.'s results [20].

SEM further confirmed the cochlear microstructure changes in the guinea pigs by showing that the noise-induced hair cell damage was mainly in the cochlear basal turn. In the normal control group, the outer and inner hair cells of the acoustic papilla were arranged in neat rows, the cilia of the outer hair cells exhibited a V-shaped arrangement, and the cilia of the inner hair cells showed a line-shaped arrangement. In the saline group, the cilia of the outer hair cells in the acoustic papilla of the basal turn exhibited sporadic absences, while those in edaravone group were neatly arranged with sporadically missing hair cellular cilia.

Noise-induced hearing loss involves a complex process that has multiple mechanisms and stages, and oxidative stress plays an important role in cochlear injuries. The noise directly damages the cochlear structure through mechanical actions, and the secondary metabolic damage-induced oxygen free radicals destroy the cellular membrane structure. In addition, as an intracellular messenger, ROS activates signaling pathways and induces apoptosis, thus leading to hearing loss. However, 7 days after noise exposure, the ABR threshold in the edaravone group still differed from that in the normal control group. This finding indicated that there may be other factors in the noise that damage the cochlear structure.

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Disclosure of conflict of interest

None.

Address correspondence to: Shi-Ming Yang and Dong-Yi Han, Department of Otolaryngology-Head and Neck Surgery, Otolaryngology Institute of PLA, General Hospital of PLA, Beijing 100853, China. Tel: +86 10 88626138; Fax: +86 10 88626138; E-mail: shimingyangcn@126.com (SMY); dongyihancn@126.com (DYH)

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