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Acoustic Stimulation Promotes DNA Fragmentation in the Guinea Pig Cochlea

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Abstract

Apoptosis can be described as programmed cell death. Apoptosis regulates cell turnover and is involved in various pathological conditions. The characteristic features of apoptosis are shrinkage of the cell body, chromatin condensation, and nucleic acid fragmentation. During apoptosis, double-stranded DNA is broken down into single-stranded DNA (ssDNA) by proteases. Acoustic trauma is commonly encountered in otorhinolaryngology clinics. Intense noise can cause inner ear damage, such as hearing disturbance, tinnitus, ear fullness, and decreased speech discrimination. In this study, we used immunohistochemical and electrophysiological methods to examine the fragmentation of DNA in the cochleas of guinea pigs that had been exposed to intense noise. Twenty-four guinea pigs weighing 250 to 350 g were used. The animals were divided into 4 groups: (I) a control group (n=6), (II) a group that was exposed to noise for 2 hours (n=6), (III) a group that was exposed to noise for 5 hours (n=6), and (IV) a group that was exposed to noise for 20 hours. The stimulus was a pure tone delivered at a frequency of 2 kHz. The sound pressure level was 120 dBSPL. No threshold shifts were apparent in group I. Group II showed a significant elevation of the hearing threshold (ANOVA, $p<0.05^{*}$). The ABR threshold level was also significantly elevated immediately after the acoustic stimulation in groups III and IV (ANOVA, $p<0.01^{**}$). In groups I, II, and IV, the lateral wall of the ear did not show immunoreactivity to ssDNA but did in group III. No immunoreactivity was apparent in the organ of Corti in group I or II. However, the supporting cells and outer hair cells in groups III and IV showed reactions for ssDNA. The fine structure of the organ of Corti had been destroyed in group IV. The lateral wall showed immunoreactivity for ssDNA only in group III, whereas the organ of Corti showed reactions for ssDNA in groups III and IV. Our study suggests that apoptotic changes occur in patients that suffer acoustic trauma. Once the apoptotic pathway has started, it is irreversible. Thus, early diagnosis and treatment are necessary. Earplugs should also be worn at rock concerts.
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Key words: apoptosis, single-stranded DNA, acoustic trauma, free radicals

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Introduction

Kerr et al.[1] have reported that in Greek "apoptosis" is used to describe the "dropping off" or "falling off" of petals from flowers and leaves from trees. More recently, it has been used to describe programmed cell death[2]. Apoptosis regulates cell turnover and is involved in various pathological conditions. In adults, the balance between cell growth and death is strictly controlled. However, various pathological conditions, such as inflammation[3] and endolymphatic hydrops[4,5], and anticancer drugs[6,7] are reported to promote apoptosis in the inner ear. The characteristic features of apoptosis are shrinkage of the cell body, chromatin condensation, and nucleic acid fragmentation. During apoptosis, double-stranded DNA is broken down into single-stranded DNA (ssDNA) by proteases[2]. Acoustic trauma is commonly encountered in otorhinolaryngology clinics. An intense noise can cause inner ear damage, such as hearing disturbance, tinnitus, ear fullness, and decreased speech discrimination[8]. We have previously reported that inducible nitric oxide synthase (iNOS) participates in the inner ear damage caused by excessive acoustic stimulation[9,10]. In this study, we used immunohistochemical and electrophysiological methods to examine the fragmentation of DNA in the cochleas of guinea pigs exposed to intense noise.

Materials and Methods

Twenty-four guinea pigs weighing 250 to 350 g were used. Preyer's reflex was confirmed to be present in all animals. Adequate anesthesia was produced with 5% (w/v) ketamine hydrochloride (50 mg/kg body weight) before the procedures. The animals were divided into 4 groups: (I) a control group (n=6), (II) a group that was exposed to noise for 2 hours (n=6), (III) a group that was exposed to noise for 5 hours (n=6), and (IV) a group that was exposed to noise for 20 hours. Kidney tissue specimens obtained after the administration of the anticancer drug cisplatin[11], were used as positive controls. The control group was used as a negative control. The protocol was in accordance with the Guidelines for Research Involving Animals and was approved by the ethics committee of our institution (No. 12–84, 13–30, 15–103, and 19–107).

Acoustic Stimulation

The head of each animal was fixed during the acoustic stimulation; however, the animals were adequately fed during this period. Acoustic stimuli were applied in a soundproof room using a sound synthesizer (Wave Factory WF1943, FF Kairo-sekkei Block Co., Kanagawa), an amplifier (Dual Power Amplifier IP600D, FF Kairo-sekkei Block Co.), and a loudspeaker (Horn Drivers D1400, Fostex, Tokyo). The loudspeaker was placed beside the animal's right ear. The stimulus was a pure tone delivered at a frequency of 2 kHz. The sound pressure level was 120 dBSPL.

Auditory Brainstem Response Measurement

Auditory brainstem responses (ABRs) were recorded with an electrodiagnostic system (Kissei Comtec Co., Ltd., Tokyo) before and after acoustic stimulation. The active electrode was inserted subcutaneously into the ipsilateral pinna, the reference electrode was inserted into the contralateral pinna, and the ground electrode was inserted into the top of the head. The animals were placed in a soundproof box, and acoustic stimuli were delivered with an earphone through a small tube inserted into the external auditory meatus. The stimuli consisted of clicks and presented at a rate of 11.1 Hz for 0.11 milliseconds. A total of 200 responses were collected. The stimuli were reduced from 103 dBSPL to 33 dBSPL in 5-dB steps. The ABR threshold was determined as the minimum sound level that produced reproducible waveforms.

Immunohistochemical Examination

All animals in each group were killed after acoustic stimulation and then used for histochemical study. The animals in the control group were killed without being exposed to noise. The animals' tissues were fixed through cardiac perfusion with 4% (w/v) paraformaldehyde. The temporal bones were immersed in the same fixative overnight. The

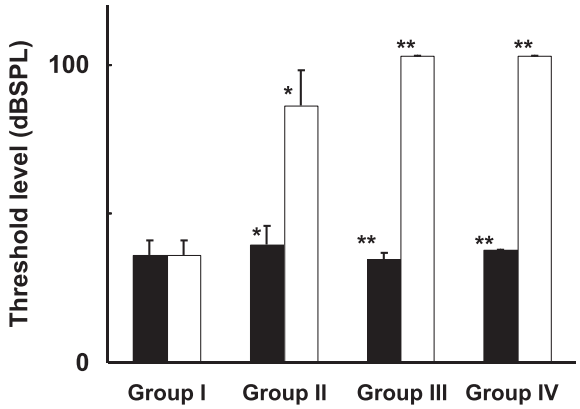

Fig. 1 The mean and SD of the threshold shift in ABR
The threshold levels observed before and after acoustic stimulation are displayed as black and white boxes, respectively. No threshold shifts were apparent in group I. Group II displayed a significantly elevated hearing threshold (ANOVA, $p<0.05$*). The ABR threshold level was significantly elevated immediately after acoustic stimulation in groups III and IV (ANOVA, $p<0.01$**).

specimens were embedded in paraffin after decalcification through incubation in a 10% EDTA solution for 5 days. Each specimen was sectioned into 8-μm-thick slices with a microtome (Leica, Bartels and Stout; Issaquah, WA, USA). After the paraffin was removed, the sections were immersed in 3% H_2O_2 for 30 minutes and then in Triton X for 10 minutes. The sections were then incubated overnight with the primary antibody to ssDNA at a 1 : 1,600 dilution (rabbit polyclonal antibody, A4506 Dako, Glostrup, Denmark). After being rinsed with Tris buffer solution and normal goat serum, the tissues were incubated with the second antibody at a 1 : 400 dilution (anti-rabbit; Dako). Development was performed with horseradish peroxidase at a 1 : 100 dilution for 1 hour and nickel-enhanced diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka).

Results

ABR Threshold Shifts

No ABR threshold shift was apparent in group I (**Fig. 1**). A significant elevation of the hearing threshold was observed in group II (ANOVA, $p<0.05$*). The ABR threshold level was also significantly elevated immediately after acoustic stimulation in groups III and IV (ANOVA, $p<0.01$**).

Hematoxylin and Eosin Staining

In group I, neither the lateral wall of the ear nor the organ of Corti showed structural degeneration on hematoxylin and eosin staining (**Fig. 2a, c**). However, 20 hours' exposure to acoustic stimulation destroyed the lateral wall of the ear and the organ of Corti in group IV (**Fig. 2b, d**).

Immunohistochemical Expression of ssDNA

The cisplatin-treated kidney tissue, which was used as a positive control, showed immunostaining of ssDNA (**Fig. 3a**). Some nuclei in the lateral wall were stained. The lateral wall did not show immunoreactivity to ssDNA in group I, II, or IV (**Fig. 3b, c, e**) but did in group III (**Fig. 3d**).

No immunoreactivity was apparent in the organ of Corti in group I or II (**Fig. 4a, b**). However, in groups III and IV the supporting cells and outer hair cells showed reactions for ssDNA (**Fig. 4c, d**). The fine structure of the organ of Corti was destroyed in group IV.

The time courses of changes in each group are shown in **Table 1**. In the lateral wall, only group III showed immunoreactivity for ssDNA, whereas in the organ of Corti, group III and IV showed reactions for ssDNA.

Discussion

In this study, we have shown that 20 hours' exposure to intense noise caused the complete destruction of the lateral wall of the ear and the organ of Corti in guinea pigs. Under physiological conditions, acoustic stimuli are conducted to the inner ear via the tympanic membrane, ossicles, oval window, tectorial membrane, and hair cells. Then, the acoustic stimuli are converted to electrical signals. Adequate sound sensation is important for daily communication and listening to music. On the other hand, intense acoustic stimulation is thought to cause metabolic and mechanical changes[12,13]. The

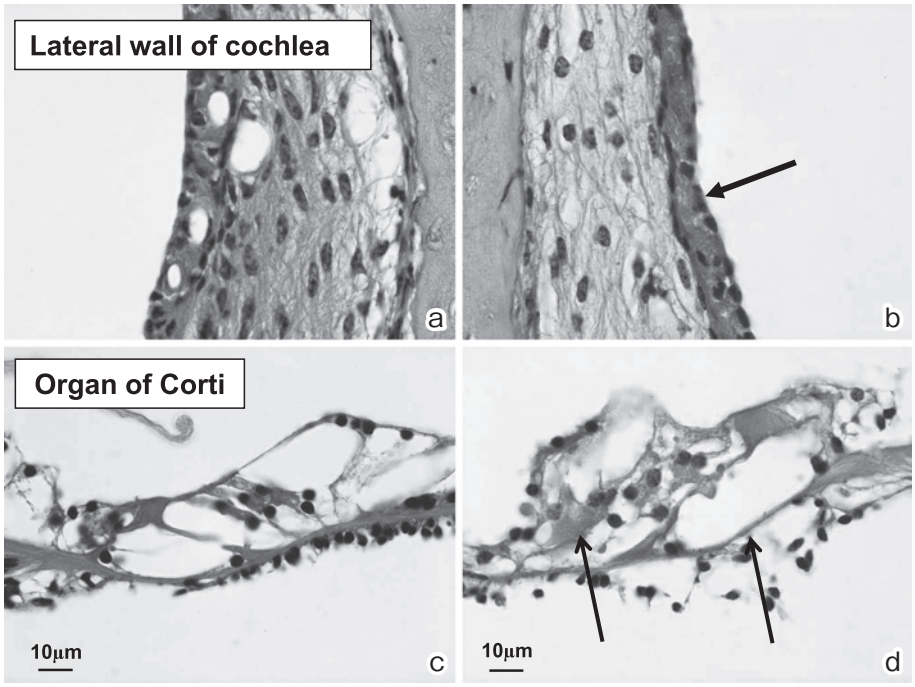

Fig. 2 Paraffin sections of the cochlea (8 μm thick). Hematoxylin and eosin.
(a) The lateral wall of the ear
In group I, the lateral wall of the ear showed no structural degeneration (×100).
(b) The lateral wall of the ear
A representative image of the lateral wall of the ear from group IV is shown. The stria vascularis was condensed and contained no apparent lumens **(arrow)** (×100).
(c) The organ of Corti
In group I, the structure of the organ of Corti was unaffected (hematoxylin and eosin staining, ×100).
(d) The organ of Corti
A representative image of the organ of Corti from group IV is shown. The supporting cells and hair cells lost have their original shapes **(arrows)** (×100).

lateral wall, especially the stria vascularis, plays a role in ion transport to maintain homeostasis in the inner ear[14,15]. When the lateral wall is damaged, inner ear disturbance occurs. The destruction of the organ of Corti is also involved in hearing disturbance.

We detected differences in ssDNA expression between groups III and IV. Group III showed ssDNA immunoreactivity in both the lateral wall and the organ of Corti; however, group IV exhibited ssDNA immunoreactivity in only the organ of Corti. Apoptosis is initiated by various stimuli, such as anticancer drugs, ultraviolet radiation, and inflammation[2,3]. Such damage activates the initiator caspases 2, 8, 9, and 10, and then the executor caspases 3, 6, and 9 induce apoptosis[2]. Finally, double-stranded DNA is broken down into DNA fragments. Our findings suggest that 5 hours' stimulation is necessary to induce apoptosis in the lateral wall, which subsequently results in the sensory organ being affected. Twenty hours' stimulation completely destroyed the cochlea. We suppose that few lateral wall cells would have survived this procedure, as the lateral wall is vulnerable to acoustic stimulation.

Free radicals have been reported to play an important role in the pathogenesis of acoustic trauma[9,10,16,17]. Intense noise stimuli directly and indirectly destroy cells in the cochlea. These damaged cells stimulate the release of cytokines, which activate the expression of free radicals. Guller et al.[18] have reported that the inhibition of iNOS reduces the hepatotoxic effects of oxide bacterial infection and endotoxins. We have also reported that iNOS is expressed in the cochlea after acoustic stimulation and that an NOS inhibitor decreases the cochlear damage produced in response to this

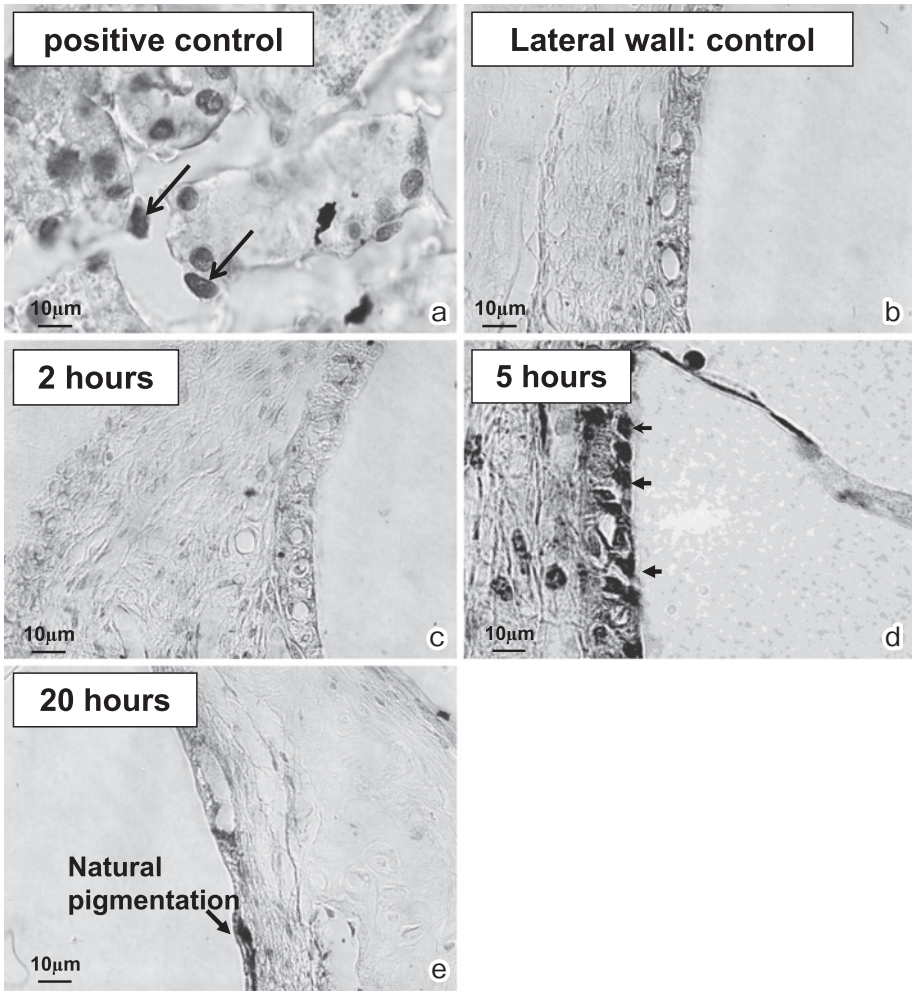

Fig. 3 Paraffin sections of the cochlea (8 μm thick). Immunohistochemical analysis using anti-ssDNA antibody.
(a) Positive control
Some nuclei in the cisplatin-treated kidney showed immunostaining for ssDNA **(arrowheads)** (×100).
(b) Control group (group I)
A representative image of the lateral wall of the ear from the control group (group I). The cochlea did not exhibit immunoreactivity for ssDNA (×100).
(c) Group II
In group II, the lateral wall of the ear did not show immunoreactivity for ssDNA (×100).
(d) Group III
Some cells in the stria vascularis showed immunoreactivity for ssDNA **(arrows)** (×100).
(e) Group IV
No ssDNA reactivity was observed in the lateral wall (×100).

stimulus[9]. Injured cells are subjected to the apoptotic pathway, which might induce DNA fragmentation in the cochlea after acoustic stimulation[13].

Significant increases in the hearing threshold were observed in groups II, III, and IV. Temporary threshold shift and permanent threshold shift are reported to be the mechanisms responsible for the hearing disturbance caused by acoustic trauma[19]. We have previously reported that 5 hours' stimulation causes a significant threshold shift; however, although the threshold shift was decreased 1 day after the stimulation it was still present 7 days after the stimulation[10]. We did not detect ssDNA in group II. Thus, the physiological damage caused by 2

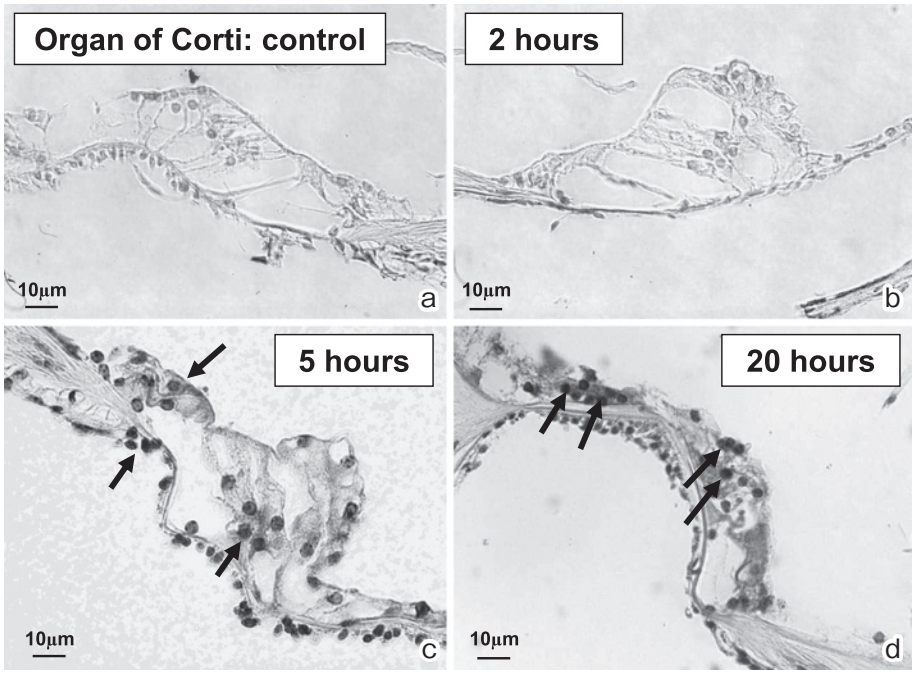

Fig. 4 Paraffin sections of the cochlea (8 μm thick). Immunohistochemical analysis using anti-ssDNA antibody.
(a) Group I
No immunoreactivity for ssDNA was detected in the organ of Corti (×100).
(b) Group II
The organ of Corti showed no immunoreactivity for ssDNA (×100).
(c) Group III
The supporting cells showed intense immunoreactivity for ssDNA **(arrows)** (×100).
(d) Group IV
The supporting cells showed intense immunoreactivity for ssDNA **(arrows)**. The structure of the organ of Corti was not maintained (×100).

Table 1 Time courses of the changes in ssDNA expression in each group

	Lateral wall of cochlea	Organ of Corti
Group I: Control	Negative	Negative
Group II: 2 hours' noise	Negative	Negative
Group III: 5 hours' noise	Positive	Positive
Group IV: 20 hours' noise	Negative	Positive

hours' acoustic stimulation might be reversible. The threshold shift observed in group II might have been due to temporary threshold shift. On the other hand, apoptotic changes were noted in groups III and IV. The threshold shifts in these groups might have been caused by a combination of temporary threshold shift and permanent threshold shift.

There are many ways to detect apoptosis. Morphological changes, such as the disappearance of microvilli from the cell membrane and the condensation of chromatin, can be detected with electron microscopy, and DNA ladders can be observed with agarose gel electrophoresis. The terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) method is an in-situ end-labeling method. We employed an immunohistochemical method targeting ssDNA, rather than the TUNEL method, for the in-situ detection of fragmented DNA with an anti-ssDNA antibody, a method that is more specific

than the TUNEL method[20]. In a previous study, DNA fragmentation was observed in cyclophosphamide-treated mouse thymic cells, and the anti-ssDNA antibody reacted specifically with DNA fragments of 200 to 300 bases in length[21]. We have previously detected ssDNA in the inner ears of guinea pigs that had been treated with cisplatin[6,7] or used as a model of endolymphatic hydrops[4,5]. We have also detected caspase 3 and caspase deoxyribonuclease using the same experimental design[4,22].

Ear protection is an effective way to avoid acoustic trauma. For example, earplugs have been reported to reduce the acoustic trauma caused by gunshots during military training[23,24]. In addition, glucocorticoids play a protective role against inner ear damage[25]. We use steroids and vitamins to treat patients who have sustained clinical acoustic trauma. However, there are no reports about therapies that protect against inner ear damage before intense noise exposure. Our study shows that apoptotic changes occur in patients with acoustic trauma. Once the apoptotic pathway starts, it is irreversible. Thus, early diagnosis and treatment are necessary. Of course, earplugs should be worn during rock concerts.

In conclusion, ssDNA was detected in the cochleas of guinea pigs exposed to acoustic stimulation, and their hearing thresholds were also elevated by the stimulation procedure. Our results suggest that apoptosis is involved in the pathogenesis of the cochlear damage caused by acoustic trauma and that protection against intense noise is necessary.

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