Cochlear Base-Apex Differences in Cell-Death Pathways Following Exposure to Low-Frequency Noise

Steve C. Lee, Barbara A. Bohne* and Gary W. Harding

Department of Otolaryngology - Head and Neck Surgery, Washington University School of Medicine, St. Louis, MO, USA

Abstract: *Objective:* To determine what death pathways are followed by noise-damaged outer hair cells (OHCs).

Methods: Chinchillas were exposed to a 0.5-kHz octave band of noise for 24 h. Administration of trypan blue *in vivo* was used to stain nuclei in cells with leaky plasma membranes. After removing unbound dye, the cochleae were fixed, plastic-embedded then dissected to obtain flat preparations of the entire organ of Corti (OC). Using morphological criteria, dying and injured OHCs were classified as following an apoptotic or oncotic death pathway, or a pathway that was neither apoptotic nor oncotic.

Results: The exposure caused scattered loss of OHCs in the apical OC and, sometimes, focal hair-cell losses in the basal OC. In the apical OC, many dying OHCs appeared to be following a death pathway that was different from apoptosis and oncosis. In the basal OC, oncotic OHCs were found at the edges of focal lesions.

Conclusion: The morphological appearance of dying OHCs in the apical OC was distinct from those following an apoptotic or oncotic death pathway, suggesting that these OHCs were following a previously unidentified death pathway.

Keywords: Cell-death pathways, noise damage, chinchilla, histopathology.

INTRODUCTION

Decreasing the magnitude of noise-induced hearing loss (NIHL) in millions of people routinely exposed to excessive noise is becoming more likely as we better understand how noise destroys cells in the cochlea and hearing ability. More than 30 million people in America are exposed to hazardous noise on a regular basis [1]. According to the National Institute on Deafness and Other Communication Disorders [2] approximately 22 million Americans have NIHL. For many years, the etiology of NIHL was thought to be the result of a combination of mechanical insult to the cells within the organ of Corti (OC) [3-5] and metabolic exhaustion of excessively stimulated hair cells [6]. More recently, it was demonstrated that free radicals accumulate in the noise-exposed cochlea [7-9]. It is unlikely, however, that all mechanisms of noise-induced cell death have been elucidated in the inner ear because of its large variety of cell types, its unique fluid spaces [10] and the differences between the cochlear apex and base with respect to function and structure.

The amount of noise-induced damage that the cochlea sustains varies with apex-to-base location in the OC. Moderate-level exposures to high-frequency noise damage cells in the high-frequency region of the cochlea (i.e., basal half of the OC). Moderate-level exposures to low-frequency noise for short durations (i.e., < 2 days) damage cells in the low-frequency region of the cochlea (i.e., apical half of the OC). However, longer exposures to low-frequency noise also damage hair cells in the basal half of the OC. The patterns of noise-induced hair-cell loss in the cochlear apex and base are different. Outer-hair-cell (OHC) loss is scattered over a broad area in the apical OC whereas basal-turn lesions begin as small, focal losses of OHCs [11-14] that have a variable distribution in the basal OC [15]. The present study was designed to test the hypothesis that exposure to moderate-level, low-frequency noise results in OHCs in the apical and basal OC following different death pathways.

Hair-cell loss often increases with post-exposure recovery time [16, 17]. The spatial-temporal component of cell death within the cochlea can be readily verified by histological examination of the entire OC at different post-exposure times.

Morphology is considered to be the gold standard for identifying cell-death pathways in a number of organ systems [18-20]. In the present study, morphology was used to identify hair cells degenerating by different death pathways. In addition, *in vivo* treatment of the cochlea with trypan blue allowed the discrimination between degenerating hair cells with intact compared to leaky basolateral plasma membranes.

MATERIALS AND METHODOLOGY

Animals

One- to two-year-old chinchillas (either sex) were purchased commercially (Ryerson Chinchilla Ranch, Plymouth, OH, USA) and maintained in a quiet animal facility prior to their use in this study. The study described here was reviewed and approved by Washington University's Animal Studies Committee (Protocol #20050314; B.A. Bohne, PI).

Four chinchillas were used for protocol development; quantitative data were not obtained from their cochleae. Three animals were used to determine hair-cell losses in non-noise-exposed cochleae; two had *in vivo* instillation of trypan

*Address correspondence to this author at the Department of Otolaryngology - Head and Neck Surgery, Washington University School of Medicine, St. Louis, MO 63110, USA; Tel: 314-362-7497; Fax: 314-362-7497; E-mail: bohneb@ent.wustl.edu

1874-4281/08

blue and one did not. Ten chinchillas were exposed to noise for 24 h. The cochleae from seven animals were processed 2-3 h post-exposure. The other three animals were allowed to recover 1 or 2 wk before cochlear processing.

Anesthesia

Before functional testing and cochlear surgery, the animals were anesthetized with a mixture of ketamine (40 mg/ml), acepromazine (1.0 mg/ml), and atropine (0.04 mg/ml), given intramuscularly at a dosage of 1 ml/kg body weight. Supplemental doses (0.5 ml/kg body weight) were given as needed throughout functional testing and surgery. Once anesthetized, the animal's eyes were liberally coated with ophthalmic ointment to prevent desiccation.

Functional Testing of Hearing: Auditory Brainstem Responses (ABRs) and Distortion Product Otoacoustic Emissions (DPOAEs)

After determining the optimal trypan-blue protocol, the remaining noise-exposed animals (#s 1016-1018, 1022 and 1024) were functionally tested. Prior to their noise exposure, the chinchillas had ABR thresholds determined in both ears using the methods described in detail previously [21]. Briefly, stainless steel needle electrodes cleaned with alcohol were inserted subcutaneously between the dorsal bullae and ventrally at the midline of the neck for the measurement of this electrophysiological potential. The animals were placed in a sound-proof booth and the sound transducers/microphone assemblies were inserted approximately 5 mm into each external ear canal.

Auditory signals consisted of 8.0 ms tone pips. The tone pips were created by the computer and transmitted to the animal *via* electronic equipment that converted the digital signal output into an acoustical pip. The equipment configuration, stimuli parameters, and determination of ABR thresholds and $2f_1$-f_2 DPOAE levels have been described previously [22]. In our chinchillas, test-retest reliability for ABR thresholds [21] and DPOAE levels [22] is ± 2 dB sound pressure level (SPL).

ABR thresholds were determined at 14 frequencies from 0.375-20 kHz. DPOAE levels were determined at 38 pairs of frequencies from 0.41-20 kHz (f_2). The f_2/f_1 ratio was 1.23 and DPOAE levels were determined at equal and unequal levels (L_2=L_1-10 dB SPL) from 65 to 45 dB SPL. Functional changes were determined by subtracting post-exposure values from pre-exposure ABR thresholds and DPOAE levels.

Noise Exposure

The exposure was an octave band of noise (OBN) with a center frequency of 0.5 kHz presented for 24 h at 95 dB SPL (free field). The exposures took place in a reverberant booth. Individual chinchillas were placed in a wire mesh cage and tethered using an aluminum collar that was attached to a wire coil running to the top of the cage. The tethering prevented the animal from lying on its side and occluding one ear canal, but at the same time, allowed freedom of movement within the cage. The noise level was adjusted to 95 ± 2 dB SPL (Brüel and Kjær sound level meter #2203) within the exposure cage. Food and water were freely available to the animals throughout their exposure.

Preparation of Trypan-Blue Solution and its Instillation into the Cochlea

Trypan blue was prepared as a 1% solution in sterile Hank's balanced salt solution (HBSS). The solution was prepared fresh and filtered just prior to use.

One cochlea of the anesthetized chinchillas was surgically exposed through an opening in the ventral bulla. In the first turn, a 200-μm diameter hole (i.e., inlet) was made caudal to the spiral ligament and stria vascularis to access scala tympani at about 80% distance from the apex. A second slightly smaller hole (i.e., outlet) was made into scala vestibuli of the third turn adjacent to the helicotrema. The inlet and outlet holes were drilled by hand with a sharpened steel pick to avoid possible noise damage from a high-speed drill. Using a small glass pipette (tip diameter ~175 μm) connected to a latex perfusion tube, a small amount of HBSS was infused into scala tympani through the inlet hole to verify that the inlet and outlet holes were patent. Patency was confirmed by observing the escape of perilymph from the outlet hole. Using another glass pipette, the trypan-blue solution was instilled into scala tympani through the inlet hole until the dye was seen escaping from the outlet hole. The solution was left in scala tympani for 5-15 minutes, and then washed out with HBSS until the fluid escaping from the outlet hole was colorless.

Cochlear Fixation, Embedding and Dissection

After removing trypan blue from the perilymphatic spaces, fixative [i.e., 1% osmium tetroxide (OsO_4) in Dalton's buffer containing 1.65% $CaCl_2$] was perfused through scala tympani from base to apex for 3 min. For some chinchillas, their second cochlea was treated in the same fashion as the first. For other chinchillas, the second cochlea was processed using our standard OsO_4 protocol [23]. These latter cochleae served as controls for fixation and to determine the toxicity of trypan blue.

After removing the temporal bones from the animal, they were washed in cold HBSS (3X - 15 min each) then immersed in 70% ethanol. Infiltration holes were made in the cochlear bone over scala tympani at the base and over scala vestibuli at the apex. The stapes footplate was removed from the oval window and most of the posterior and superior semicircular canals were trimmed away. These openings into the cochlea were created to allow adequate fluid exchange between the cochlear scalae and the specimen vial during subsequent steps of dehydration and plastic infiltration. Care was taken to avoid mechanically violating the endolymphatic space, basilar membrane and OC.

The cochleae were stored overnight in a refrigerator after which they were inspected for bubbles and bone chips. While immersed in 70% ethanol, the cochleae were repeatedly rotated from dorsal to ventral side so that any bubbles and bone chips in the perilymphatic spaces were shifted close to the basal infiltration hole or the oval window. At this point, the bubbles and bone chips were removed using very light suction with a glass pipette. The cochleae were dehydrated in increasing concentrations of ethanol (i.e., 80, 95, 100 and 100% - 0.5 h per solution), then in propylene oxide (2 changes - 0.5 h each). The process of dehydration was facilitated by use of a bottle rotator (Electron Microscopy

Sciences, #71780-10) which improved fluid exchange between the liquid in the cochlear scalae and that in the specimen vial.

The cochleae were infiltrated with propylene oxide and araldite mixtures (3 dilutions - 0.5 h each), and finally 100% araldite (4 changes - 1 h each). A 100-W light bulb was shined on the vials in the bottle rotator when the specimens were in pure plastic. The heat from the bulb reduced the viscosity of the plastic and increased the rate of propylene oxide evaporation from the specimen. Once infiltrated, the cochleae were placed in a 60^{o} C oven for 48 h to polymerize the plastic.

After polymerization, the surrounding plastic and cochlear bone were removed with razor blades and a sharpened steel pick and the cochlear ducts were divided with pieces of razor blade into 17-22 'segments', each containing a variable length of the OC. After trimming each segment close and parallel to the basilar membrane, they were re-embedded in thin layers of plastic for observation by phase-contrast and bright-field microscopy (Wild M-20) [23]. By dissecting the plastic-embedded cochlea after plastic polymerization, cellular debris in the fluid spaces of the OC and the endolymphatic space remained within the specimen at or near its *in vivo* location.

Quantitative and Qualitative Histological Evaluation

For each cochlea, OC length was determined by acquiring images of the OC segments using a digital camera (Qimaging Micropublisher 5.0, Burnaby, BC, Canada) mounted on a dissection microscope (Nikon SMZ-U). The images of each segment were displayed on a computer monitor using the NIH-developed ImageJ software package. This system was calibrated at a specific microscope magnification by determining the precise number of pixels per 1 mm length on a hemocytometer. Using ImageJ, a line was drawn across each OC segment at the approximate junction of the inner and outer pillar heads. The length of this line was determined by the software and summed together with the lengths of the other OC segments to determine the overall length of the OC in millimeters for that cochlea.

Hair cells were assessed by focusing at sequential levels (i.e., optical sectioning) through each OC segment using a phase-contrast microscope at a magnification of 1250X. By focusing back and forth from the hair-cell apices to their bases without changing the x and y controls on the microscope, an individual cell could be followed from its stereocilia through the cuticular plate to its nucleus (e.g., Fig. (**1**)). With intact OHCs, the basolateral plasma membrane and peripheral membrane system appeared as a heavy line from just below the cuticular plate to just above the nucleus. Severely damaged OHCs had intact plasma membranes and were shrunken and dark with a basal nucleus (i.e., apoptotic) or were swollen and pale with a central nucleus (i.e., oncotic). Degenerating OHCs were either grossly swollen with a swollen, pale nucleus near the cell's center or had a normal shape and a nucleus that was normal to slightly swollen, pale and basally located. In these latter two instances, the OHCs were missing all or most of their basolateral plasma membrane. The grossly swollen cells fit the description of necrosis while the normal-shaped cells each had a narrow strip of cellular debris beneath the cuticular plate and appeared to be following a death pathway that was distinct from apoptosis and oncosis [24]. The cellular and nuclear characteristics, including *in vivo* staining with trypan blue (see below) of these different classes of pathological cells are summarized in Table **1**.

Missing hair cells in the OC were replaced by either mature phalangeal scars (i.e., dark line of junction between phalangeal processes) or immature phalangeal scars (i.e., pale line of junction between phalangeal processes) [24, 25]. If no cell debris was found in the OC at the location of a mature or an immature scar, the missing cell was classified as having followed an unknown death pathway. In these latter instances, death pathway could not be determined because no pathological clues remained (e.g., apoptotic bodies, nuclear, cytoplasmic and/or plasma membrane fragments).

Special attention was paid to the distribution of blue-stained nuclei within the OC. Stained nuclei could be identified and counted in the flat preparations by either phase-contrast or bright-field microscopy. However, decreasing ambient light in the room and using phase-contrast microscopy improved our ability to identify the cell body, cell debris or cell apex to which the blue-stained nucleus belonged.

When hair-cell loss was concentrated in part of a dissected OC segment, that segment was mathematically divided into subdivisions, on the basis of inner hair cell (IHC) density, so that the position(s) of 'focal lesion(s)' could be preserved in the individual cytocochleograms. A focal hair-cell lesion has been defined as an OC region in which the loss of IHCs, OHCs or both cell types is 50% or more over a distance of at least 0.03 mm [13, 26]. These concentrated areas of hair-cell loss were bordered by regions of substantially less hair-cell loss.

For each OC segment or subdivision in a particular cochlea, the total number of each hair-cell type that was missing (i.e., mature or immature phalangeal scars), degenerating

Table 1. Morphological Characteristics of Severely Damaged and Dying OHCs

Death Pathway	Cell Characteristics			Nuclear Characteristics		
	Size	OsO_4-Stain	Plasma Membrane	Size	OsO_4-Stain	Trypan Blue
Apoptotic	Shrunken	Dark	Intact	Shrunken	Dark	Negative
Oncotic	Swollen	Pale	Intact	Normal	Normal to pale	Negative
Necrotic	Grossly swollen	Very pale	Disrupted	Swollen	Very pale	Positive
Non-apoptotic, & non-oncotic	Normal	Stippled	Disrupted	Normal to slightly swollen	Pale & granular	Positive

(i.e., necrotic or non-apoptotic, non-oncotic) and severely damaged (i.e., apoptotic or oncotic) was summed. These data were entered into a computer program along with the millimeter length of that segment or subdivision. The computer program calculated the expected total number of IHCs and OHCs per row in a particular segment or subdivision, based on equations that describe the relation between hair-cell density and total length of the particular OC, and apex-to-base location of the segment [27]. The percentages of missing IHCs and OHCs were calculated by dividing the sum of missing and damaged hair cells by the calculated total number of hair cells for that segment or subdivision. These data were compiled into a cytocochleogram for that particular cochlea. Conversion to hair-cell-loss percentages as a function of percentage distance from the OC apex was necessary to compensate for variations in OC length among animals and hair-cell density within and across cochleae [27-29].

Although the 0.5-kHz OBN supplies maximal energy to the basilar membrane at 0.375-0.750 kHz, hair-cell losses from a moderate exposure to this noise can occur anywhere in the apical half of the OC. Longer exposures to the 0.5-kHz OBN produce additional lesions in the basal half of the OC. These latter hair-cell losses are morphologically indistinguishable from those produced by exposure to a 4-kHz OBN [11, 30]. The hair-cell-loss percentages were summarized for the apical OC (0-50% distance from the apex) and basal OC (50.1-100% distance) to permit statistical analysis of hair-cell losses in cochleae in the < 3-h, 1- and 2-wk recovery groups.

Statistical Analysis

OHC losses were analyzed using a two-way ANOVA. The first independent variable was death pathway (non-apoptotic, non-oncotic death pathway versus oncotic pathway plus apoptotic pathway). We combined those cells degenerating by the oncotic and apoptotic pathways in order to simplify the analysis; using three separate groups yielded similar results. The second independent variable was cochlear location (i.e., apical OC versus basal OC).

RESULTS

Trypan-Blue Protocol

In vivo treatment with trypan blue for 5 or 10 min did not result in clear nuclear staining in degenerating hair cells in the noise-exposed cochlea. Fixation of the cochlea prior to washing free dye out of scala tympani resulted in the staining of nuclei in both healthy and degenerating cells in the OC. *In vivo* treatment with trypan blue for 15 min followed by removal of unbound dye prior to fixation resulted in selective staining of a variable number of degenerating hair cells in the noise-exposed cochleae (e.g., Figs. (**1**,**2**)).

Cochlear Fixation

The duration of fixation was varied to optimize OC preservation yet still allow clear visualization of cell shape and the presence and distribution of trypan-blue staining. Fixation for 3 min did not provide adequate OC preservation, especially the OHCs. In addition, cell membranes were difficult to visualize. A 2-h fixation interval produced excellent preservation but it was very difficult to identify trypan-blue staining in the OC. Fixation for 8 min was found to result in adequate hair-cell fixation without masking trypan-blue stained nuclei in the OC.

Hair-Cell Losses and Trypan-Blue Staining

Losses of OHCs and IHCs were determined in the apical OC and basal OC for controls and noise-exposed chinchillas with < 3-h, 1- or 2-wk recovery. The losses in Table **2** are expressed in percentage of the total hair cells in the apical and basal OC.

Non-Noise-Exposed Cochleae

Three cochleae were used to determine the magnitude of loss and the death pathways followed by hair cells in non-noise-exposed controls. In these cochleae, OHC and IHC losses averaged 2.2% and 0.7%, respectively, in the apical OC and 0.8% and 0% in the basal OC (Table **2**). None of the controls had a focal hair-cell lesion. Most hair cells that were missing in these cochleae had been replaced by mature phalangeal scars [24]; there was no cell debris in the OC associated with the scars. It is likely that the loss of OHCs in controls took place over 1-2 years of the animals' lives rather than just prior to sacrifice. Therefore, most of the missing cells were classified as having degenerated by an unknown death pathway (Table **3**).

In cochlea #1015L (no trypan blue), there were no degenerating OHCs but there were a few immature scars near the apex. The percentages of missing hair cells in cochlea #1015L were similar to those in cochleae #1019R and #1020L (both instilled with trypan blue). In cochlea #1019R, two trypan-blue-stained OHC nuclei were observed in the apical OC while no blue-stained nuclei were observed in cochlea #1020L.

Noise-Exposed Chinchillas with < 3-h Recovery

The cochleae from seven chinchillas were fixed for microscopy < 3-h post-exposure. OHC and IHC losses averaged 8.0% and 2.3%, respectively, in the apical OC (Table **2**). The loss of apical OHCs was significantly greater than in controls ($p < 0.05$). Because mature phalangeal scars replaced some missing OHCs in the apex, these OHCs must have degenerated prior to the noise exposure. However, because there were a number of immature phalangeal scars and some degenerating OHCs (Fig. **1**) in the apical OC, many OHCs must have died as a result of the exposure. Many of the degenerating OHCs had appearances that were morphologically distinct from cells that were apoptotic or oncotic in the same cochleae. In addition, these cells contained nuclei that were pleomorphic in appearance and were stained with trypan blue (Figs. **1**,**2**). These OHCs were difficult to document photographically because their bodies lacked an intact basolateral plasma membrane and consisted of sparse cellular debris and a pale, granular nucleus only. However, these cells could be identified by careful optical sectioning of the flat preparations (Fig. **1**).

Sometimes, blue-stained nuclei were found beneath immature phalangeal scars. The number of dead and dying OHCs with blue nuclei ranged from 0–201 in the apical OC across the nine cochleae that were evaluated quantitatively in this group. In these same cochleae, fewer OHCs were apoptotic or oncotic (Table **3**) and none of these latter cells contained trypan-blue-stained nuclei.

Table 2. Combined Percentage of Missing[a] Hair Cells and Focal Hair-Cell Lesions in the Apical OC and Basal OC

Ear / Recovery	Apical OC (0-50% Distance from Apex)			Basal OC (50.1-100% Distance from Apex)		
	%mOHC	%mIHC	Focal Lesion Size, Type and % Location	%mOHC	%mIHC	Focal Lesion Size, Type and % Location
1015L (control)	1.5	0.7	None	1.0	0	None
1019R (control)	3.0	1.0	None	1.3	0.1	None
1020L (control)	2.1	0.4	None	0.2	0	None
Mean ± S.D.	**2.2 ± 0.8**	**0.7 ± 0.3**		**0.8 ± 0.6**	**0.0 ± 0.1**	
1010R (< 3 h)	4.2	0.4	None	1.4	2.0	None
1011R (< 3 h)	3.5	0.3	None	6.9	0.2	0.29 mm OHC @ 65%
1012R (< 3 h)	6.9	6.7	None	0.2	0.2	None
1013R (< 3 h)	11.6	2.0	None	1.0	1.2	None
1014L (< 3 h)	18.6	1.8	1.15 mm OHC @ 16%; 0.53 mm OHC @ 22%	1.9	1.7	0.18 mm OHC @ 50%
1016L (< 3 h)	9.1	1.4	None	0.1	0.1	None
1016R (< 3 h)	7.4	6.7	None	0.6	0	None
1022L (< 3 h)	6.1	0.3	None	0.3	0	None
1022R (< 3 h)	4.5	0.9	None	0.3	0	None
Mean ± S.D.	**8.0 ± 4.7**	**2.3 ± 2.6**		**1.4 ± 2.1**	**0.6 ± 0.8**	
1017L (2 wk)	2.9	0.3	None	1.3	0.2	None
1017R (2 wk)	4.9	0.2	None	1.6	0.2	None
1018L (2 wk)	21.9	4.0	0.35 mm O/IHC @ 26%	1.3	3.3	0.12 mm IHC @ 67%
1018R (2 wk)	21.8	6.8	0.71 mm O/IHC @ 21%; 0.07 mm IHC @ 48%	1.8	3.2	0.11 mm IHC @ 86%
1024R (1 wk)	6.1	2.0	0.03 mm IHC @ 25%	3.1	0.6	0.27 mm OHC @ 66%; 0.06 mm OHC @ 98%
Mean ± S.D.	**11.5 ± 9.5**	**2.7 ± 2.8**		**1.8 ± 0.7**	**1.5 ± 1.6**	

[a]Includes phalangeal scars and cells dying by the necrotic, apoptotic, oncotic and non-apoptotic, non-oncotic death pathways.

In the basal OC, OHC and IHC losses averaged 1.4% and 0.6%, respectively. Two of nine cochleae (i.e., #s 1011R; 1014L; Table **2**) had a basal focal lesion. Within the focal lesions, three OHC nuclei were stained with trypan blue in #1011R and one OHC nucleus was stained in #1014L. Oncotic OHCs were present at the borders of these lesions (Fig. **3**), but their nuclei were not stained with trypan blue. In the larger focal lesion (Fig. **4**), many OHCs had already degenerated when the cochlea was fixed. The degenerated cells left behind cellular debris in the fluid spaces. In the same lesions, there were very few nuclei and no recognizable nuclear debris. Cytocochleograms from cochleae with and without basal focal lesions are shown in Figs. (**5**,**6**), respectively.

Noise-Exposed Chinchillas with 1- or 2-wk Recovery

OHC and IHC losses averaged 11.5% and 2.7%, respectively, in the apical OC (Table **2**). These losses were significantly greater than those in controls ($p < 0.05$) but not significantly different from those in the < 3-h recovery cochleae. Many of the degenerated OHCs had been replaced by mature phalangeal scars. However, a variable number of immature scars was present, along with a few degenerating OHCs. Only two of five cochleae had a moderate number of oncotic OHCs in the apical OC (Table **3**). A few degenerating OHCs were neither apoptotic nor oncotic and some of these cells had blue-stained nuclei (Fig. **7**). Much cellular debris was present in the fluid spaces of the OC but generally, there were no degenerating nuclei, recognizable nuclear debris, or blue-stained nuclei. Three of five cochleae (i.e., #s 1018L, 1018R, 1024R; Table **2**) had recent basal focal lesions as evidenced by degenerating hair cells, hair cells with blue-stained nuclei or degenerating nerve fibers in the osseous spiral lamina. There were a variable number of oncotic OHCs in these focal lesions, but none had blue-stained nuclei. The cytocochleogram from a cochlea that was processed 2-wk post-exposure is shown in Fig. (**8**). In cochlea #1024R, there was an OC region at 66% distance from the apex that contained 12 degenerating OHCs with blue-stained nuclei. This lesion had 21% OHC loss which did not reach the 50% minimum that defines a focal lesion.

Statistical Analysis of OHC Losses by Death Pathway

OHC loss was evaluated quantitatively in the apical OC and basal OC by categorizing all severely damaged and

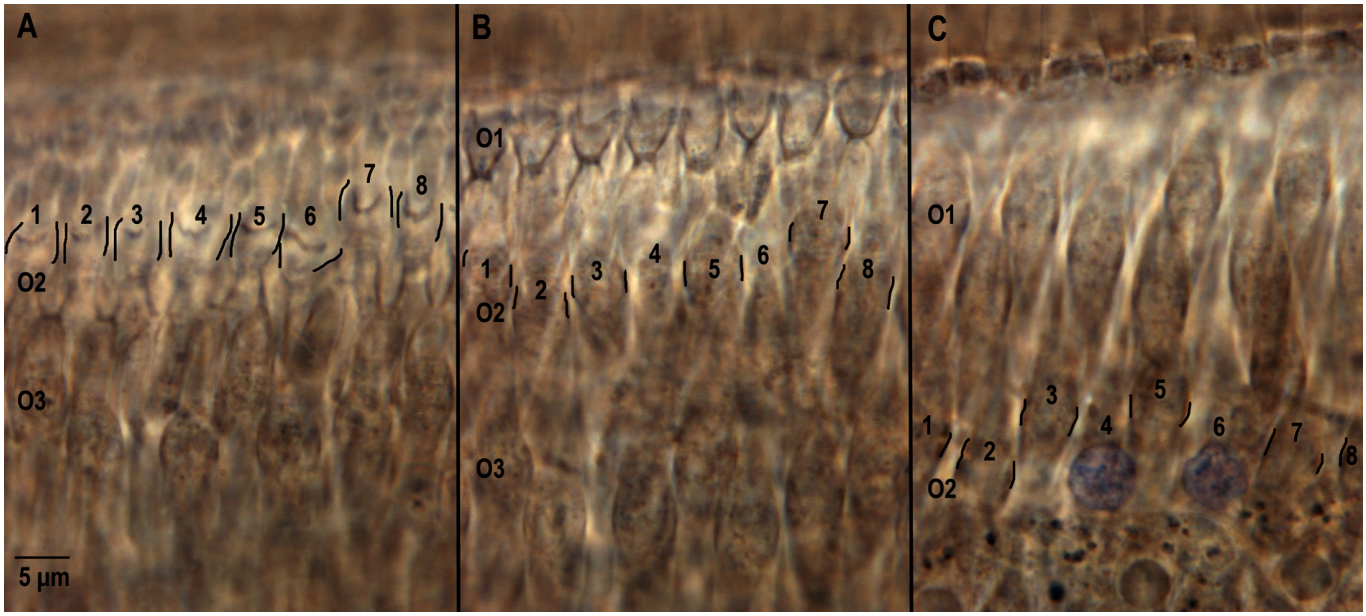

Fig. (1). Phase-contrast appearance of #1012R (< 3-h recovery) at 24% distance from apex. OHCs in 2nd row (O2) are partly outlined in black and have same numbers in each panel, representing different OC focal planes. **A**) At reticular lamina, eight cuticular plates (1-8) are visible in 2nd row. OHCs 1-3, 5 and 7-8 are similar in size while OHCs 4 and 6 have enlarged apices and a pale cuticular plate (4) or a distorted stereocilia bundle (6); **B**) OHC bodies 1-3, 5 and 7-8 have clearly defined basolateral plasma membranes. OHC bodies 4 and 6 consist of a narrowed strip of cellular debris and lack a plasma membrane; **C**) Just above O2 nuclei, OHCs 1-3, 5 and 7-8 have a distinct plasma membrane while a trypan-blue-stained nucleus not surrounded by a plasma membrane is positioned directly beneath cell apices 4 and 6 in 'A' and cell debris in 'B'. O1, O3 - 1st and 3rd rows of OHCs.

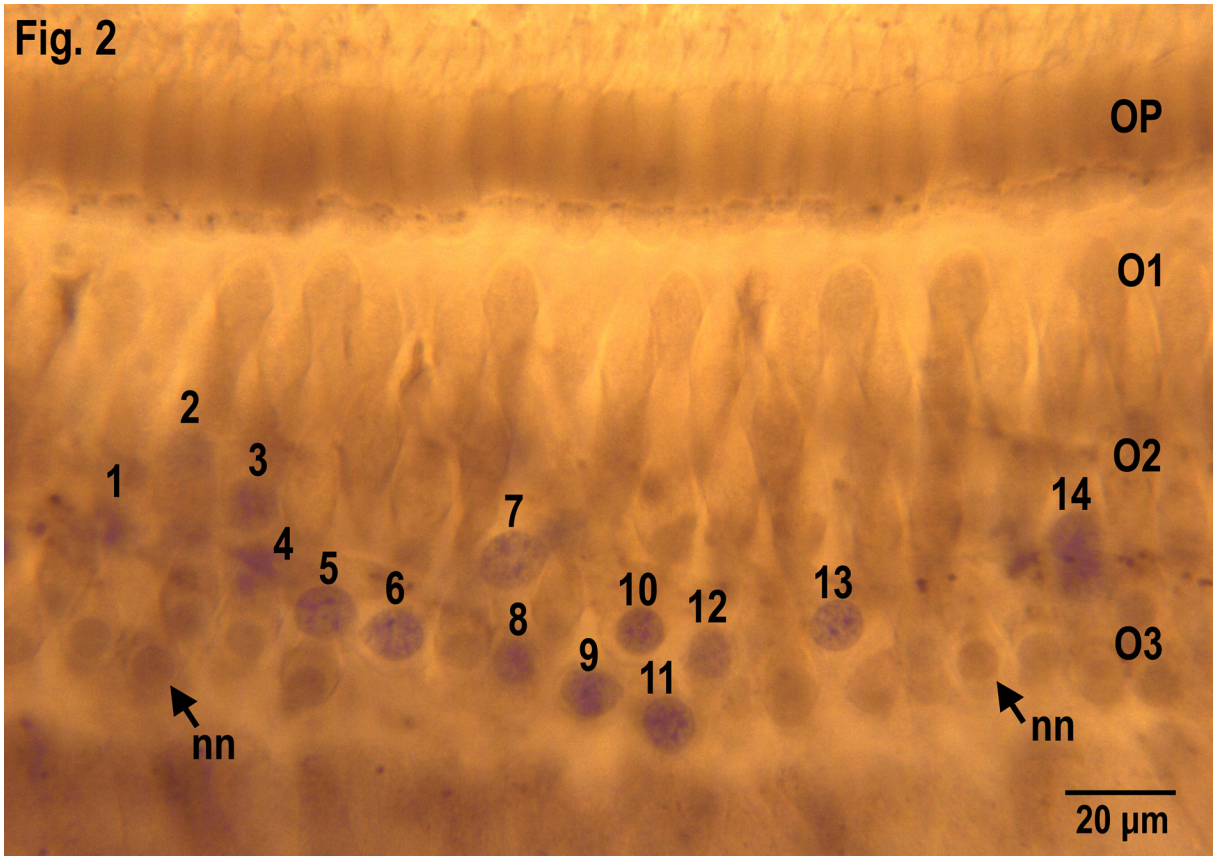

Fig. (2). Bright-field view of #1012R at 21% distance from apex. Fourteen trypan-blue-stained nuclei are visible. These nuclei are pleomorphic. Some are pale-stained with few granules; others are dark-stained with many granules. Many are normal-sized; some are slightly swollen. OP - Outer pillar heads; nn - normal-sized nuclei not stained with trypan blue.

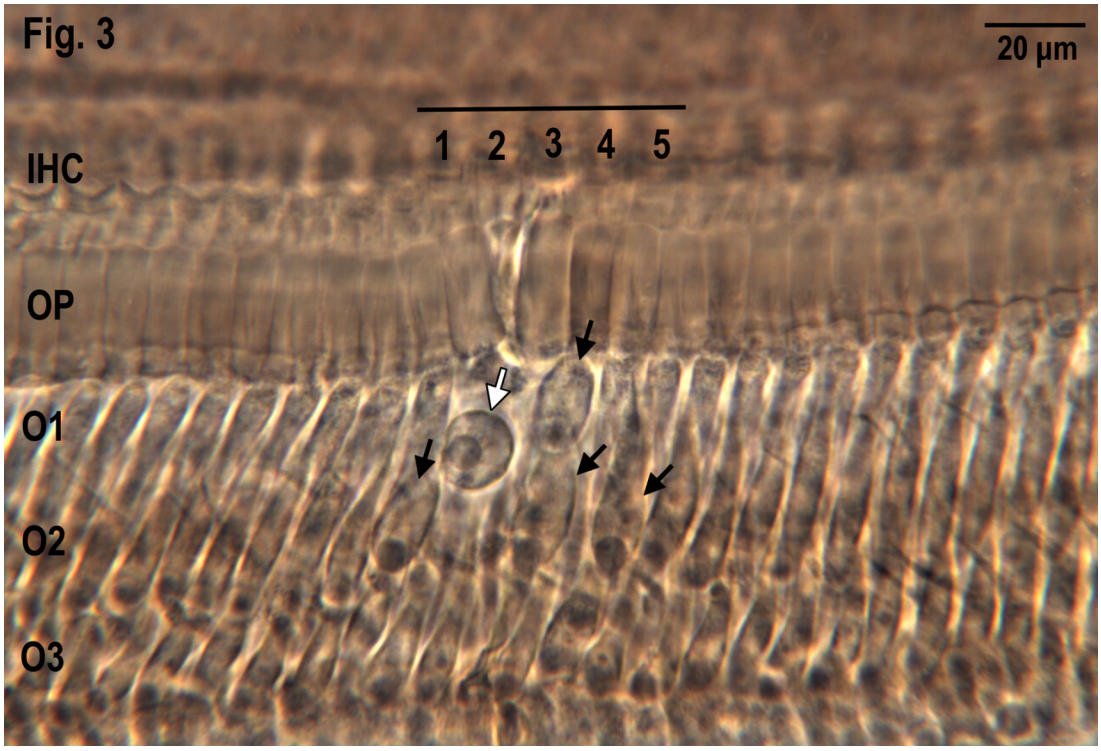

Fig. (3). Phase-contrast appearance of #1011R (< 3-h recovery) at 60% distance from apex. A 0.06-mm OHC lesion that spans the distance of five inner hair cells (IHCs 1-5) is located under black bar. In lesion, one oncotic OHC (white arrow), several slightly swollen OHCs (black arrows) and one missing outer pillar (OP; clear space) are seen. The oncotic OHC does not have a trypan-blue-stained nucleus. O1, O2, O3 - Outer hair cells in 1st, 2nd and 3rd rows.

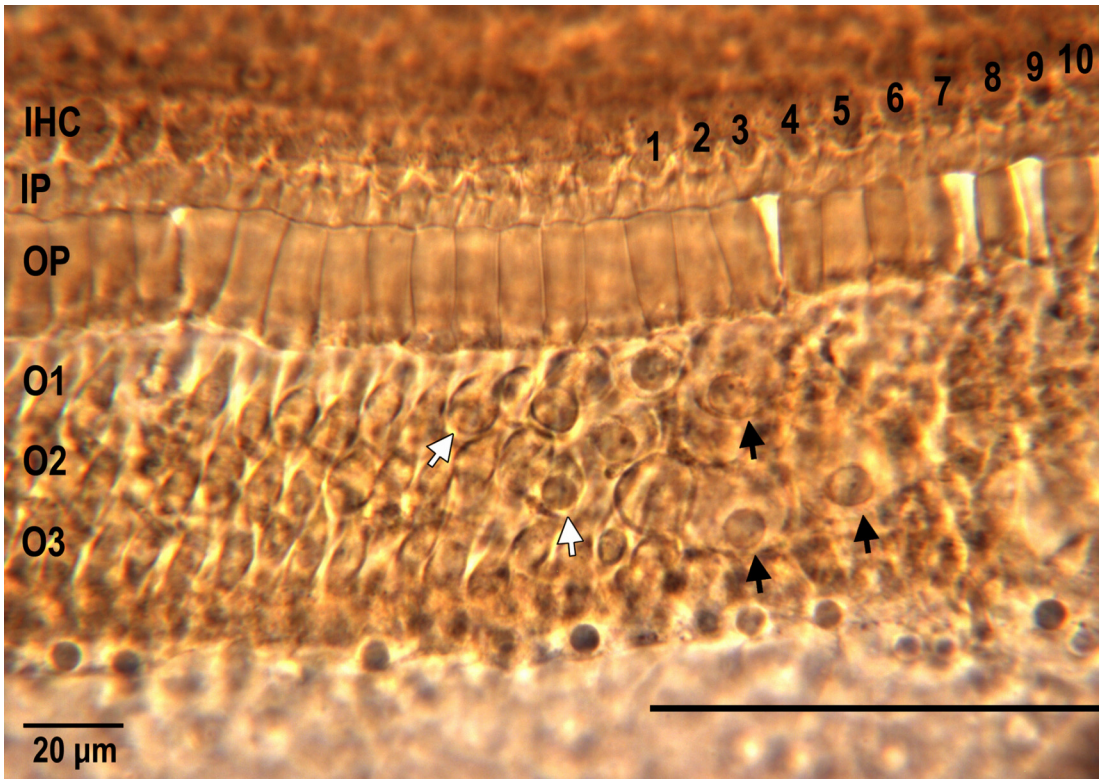

Fig. (4). Phase-contrast appearance of #1011R at 64% distance from apex. Apical edge of large focal OHC lesion (horizontal black bar) is near middle of field. This lesion spanned 26 inner hair cells (IHC), 10 of which are numbered here. Apical to lesion (i.e., left), most IHCs and OHCs in 1st (O1), 2nd (O2) and 3rd (O3) rows are present. Some OHCs are slightly swollen (white arrows). Near apical edge, several necrotic OHCs (black arrows) with grossly swollen bodies, enlarged pale nuclei and ruptured plasma membranes are seen. Three missing outer pillars (OP) appear as clear spaces in line of OP heads. IP - inner pillar heads.

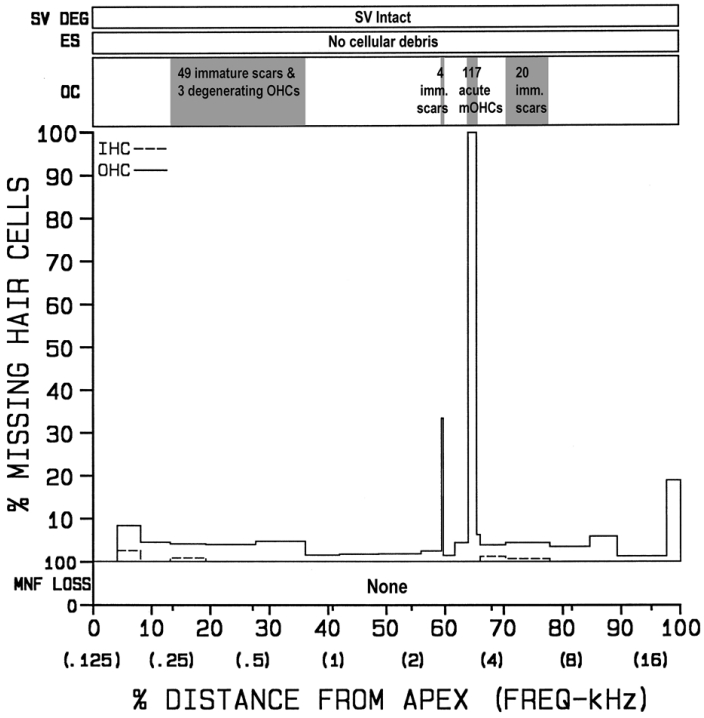

Fig. (5). Cytocochleogram for #1011R (< 3-h recovery). As function of % distance from apex and frequency place (x-axis), 5 y-axis boxes (top to bottom) indicate: 1) **SV DEG** - Location of degenerated regions of stria vascularis; 2) **ES** - Cellular debris in endolymphatic space; 3) **OC**- Number and locations of immature phalangeal scars, degenerating and acutely missing OHCs are shown in gray boxes; 4) **% missing OHCs and IHCs**, includes recent and longstanding hair-cell losses; 5) **MNF LOSS** - % loss of myelinated processes of spiral ganglion cells. There were 3.5% missing OHCs and 0.3% missing IHCs in apical OC, a 0.06-mm OHC lesion at 60% (Fig. **3**) and a 0.29-mm focal OHC lesion centered at 64% distance from apex (Fig. **4**).

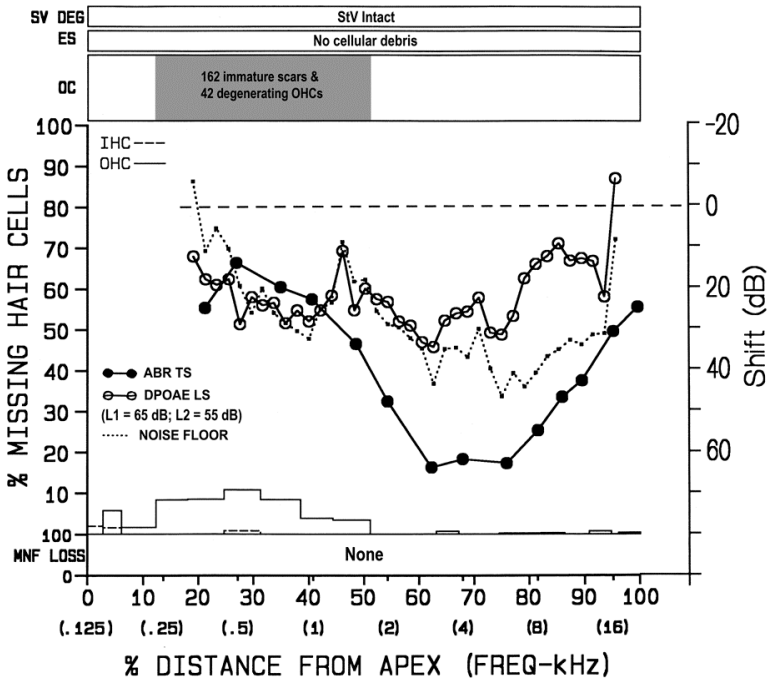

Fig. (6). Cytocochleogram for #1022L (< 3-h recovery) with functional overlay aligned by frequency-place map [31]. Right y-axis shows animal had ABR threshold shift (TS; solid circles) of 25-63 dB from 0.375-20 kHz and DPOAE level shift (LS; open circles) of 13-36 dB from 0.5-13 kHz, compared to pre-exposure data (horizontal dashed line). Dotted line represents differential noise floor for DPOAE LSs. OHC loss was 6.1% in apical OC and there was no basal focal lesion. Number and location of degenerating OHCs and immature phalangeal scars are shown in gray area of OC box.

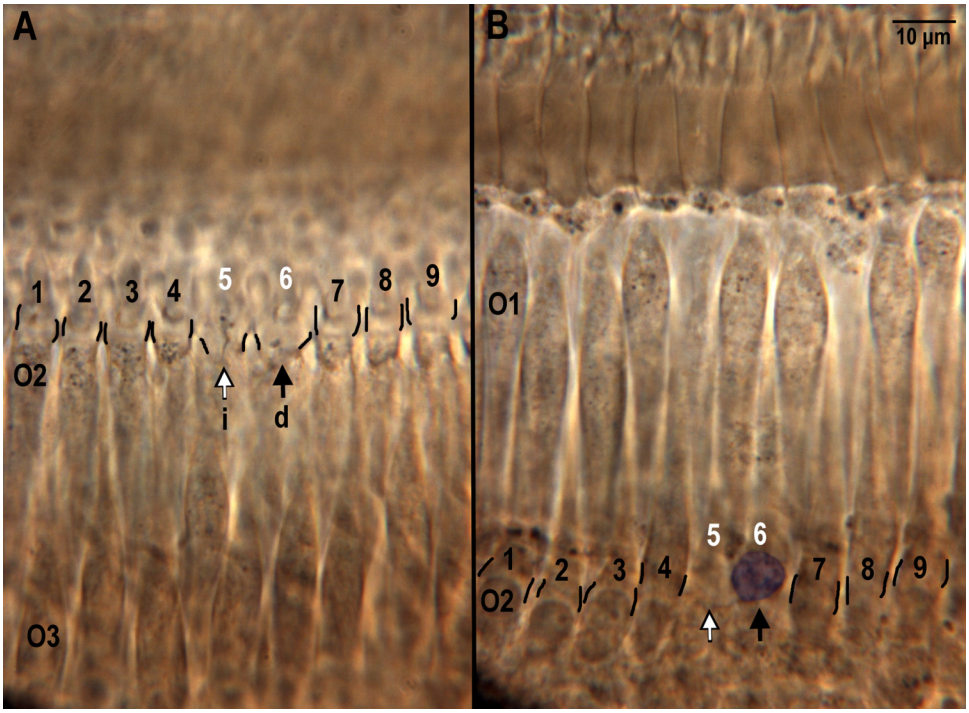

Fig. (7). Phase-contrast appearance of #1017R (2-wk recovery) at 17% distance from apex. OHCs in 2nd (O2) row are partly outlined in black and are numbered identically in panels A and B which are two focal planes in same OC region. **A**) At reticular lamina, OHCs 1-4 and 7-9 have similar-sized cuticular plates. An immature phalangeal scar (i) is located at 5; the enlarged cuticular plate of a degenerating OHC (d) is located at 6; **B**) Focused on basolateral plasma membrane of O2 cells, slightly above nuclei. OHCs 1-4 and 7-9 have intact basolateral membranes and normal nuclei (some slightly out-of-focus). Beneath immature scar (i) in 'A', there is no cell body or nucleus (white arrow). Beneath enlarged cuticular plate (d) in 'A', there is an enlarged, trypan-blue-stained nucleus (black arrow). O1, O3 - bodies of 1st and 3rd row OHCs.

degenerating OHCs and phalangeal scars by death pathway (Table **3**). The death pathways followed by missing OHCs that were replaced by mature phalangeal scars could not be determined and are listed as 'unknown' in Table **3**. Our null hypothesis was that OHCs degenerating by a death pathway other than apoptosis or oncosis occur with the same frequency in the apical and basal OC after exposure to the 0.5-kHz OBN. Hair-cell losses in focal lesions were not included in this analysis because focal lesions were not consistently found in these cochleae. The mean number of degenerating hair cells found in control cochleae (i.e., 1 apoptotic; 1 oncotic; 7 non-apoptotic, non-oncotic) was subtracted from the counts in the noise-exposed cochleae.

In the statistical analyses, both main effects were significant. In the < 3-h recovery and 1- or 2-wk recovery cochleae (apical and basal halves combined), more OHCs followed a death pathway that was morphologically distinct from apoptosis and oncosis (mean: 167 ± 108 cells) than those dying by apoptosis and oncosis combined (15 ± 13 cells) ($p < 0.00002$). For all three death pathways, more OHCs died in the apical OC compared to the basal OC (173 ± 115 versus 10 ± 15 cells; $p < 0.00001$). Importantly, the interaction was significant in that the relative frequency of cells degenerating by the non-apoptotic, non-oncotic death pathway compared to those degenerating by apoptosis plus oncosis depended on location in the cochlea ($p < 0.00005$).

Functional Losses Compared to Structural Damage

Shortly after exposure, ABR threshold shifts (TSs) were broad, covering a frequency range of 0.375-20 kHz (Table **4**). Threshold shifts ranged from 15-66 dB, peaking at 4.3 kHz in the basal OC. The ABR TSs were remarkably similar across chinchillas. DPOAE level shifts (LSs) immediately post-exposure were also broadly distributed over a slightly narrower frequency range compared to the ABR TSs. Level shifts ranged from 10-61 dB over 0.6-13.5 kHz, peaking at 4.4 kHz, similar to the frequency of the maximum ABR TS.

In general, ABR TSs nearly recovered across all frequencies, except a residual 10-12 dB TS at 6-16 kHz in three of five 1- or 2-wk recovery cochleae (Fig. **8**, Table **4**). Likewise, DPOAE LSs fully recovered at nearly all frequencies. There was no correspondence between ABR TSs, DPOAE LSs and the frequency-place of focal lesions in the < 3-h, 1- or 2-wk recovery cochleae.

DISCUSSION

Distinguishing Cell-Death Pathways

The morphological characteristics of cells following the apoptotic and oncotic (i.e., necrotic) death pathways are quite similar in a variety of tissues from a number of species. Cells following the apoptotic death pathway are shrunken and dark-staining and have pyknotic nuclei with chromatin

Table 3. Number (and Percentage) of Total OHCs Following Different Death Pathways in the Apical and Basal OC

Ear # / Recovery	Apical Half of OC (0-50% Distance from Apex)				Basal Half of OC (50.1-100% Distance from Apex)			
	Apoptotic	Oncotic [Necrotic]	Non-Apoptotic, & Non-Oncotic	Unknown	Apoptotic	Oncotic [Necrotic]	Non-Apoptotic & Non-Oncotic	Unknown
1015L (control)	0	0	5 (9%)	50 (91%)	0	0	0	35 (100%)
1019R (control)	4 (3%)	2 (2%)	8 (7%)	104 (88%)	0	0	2 (4%)	45 (96%)
1020L (control)	0	0	9 (11%)	74 (89%)	0	0	1 (11%)	8 (89%)
Mean ± S.D.	**1 ± 2**	**1 ± 1**	**7 ± 2**	**76 ± 27**	**0**	**0**	**1 ± 1**	**29 ± 19**
1010L (< 3 h)	27 (13%)	0	81 (40%)	95 (47%)	2 (4%)	0	10 (19%)	41 (77%)
1011R (< 3 h)	32 (23%)	0	67 (48%)	41 (29%)	2 (1%)	1 [111][a] (42%)	53 (20%)	96 (37%)
1012R (< 3 h)	31 (11%)	0	213 (79%)	26 (10%)	0	0	5 (63%)	3 (37%)
1013R (< 3 h)	4 (1%)	6 (1%)	420 (80%)	92 (18%)	4 (11%)	0	2 (5%)	31 (84%)
1014L (< 3 h)	0	6 [460][a] (63%)	203 (27%)	72 (10%)	1 (2%)	0	16 (30%)	36 (68%)
1016L (< 3 h)	28 (8%)	0	304 (84%)	29 (8%)	0	0	2 (67%)	1 (33%)
1016R (< 3 h)	28 (9%)	1 (0%)	243 (81%)	29 (10%)	0	0	3 (15%)	17 (85%)
1022L (< 3 h)	0	2 (1%)	173 (85%)	28 (14%)	0	0	0	6 (100%)
1022R (< 3 h)	14 (8%)	3 (2%)	135 (79%)	19 (11%)	0	0	1 (25%)	3 (75%)
Mean ± S.D.	**18 ± 14**	**2 ± 3**	**204 ± 111**	**48 ± 30**	**1 ± 1**	**0 ± 0**	**10 ± 17**	**26 ± 30**
1017L (2 wk)	0	0	10 (9%)	106 (91%)	0	0	0	48 (100%)
1017R (2 wk)	0	0	77 (40%)	116 (60%)	0	0	0	59 (100%)
1018L (2 wk)	0	0 [139][a] (17%)	71 (8%)	629 (75%)	1 (2%)	0	1 (2%)	44 (96%)
1018R (2 wk)	1 (0%)	2 [289][a] (35%)	144 (17%)	403 (48%)	0	0	2 (3%)	70 (97%)
1024R (1 wk)	0	0	89 (41%)	127 (59%)	0	11 [18][a] (26%)	16 (14%)	66 (59%)
Mean ± S.D.	**0 ± 0**	**0 ± 1**	**78 ± 48**	**276 ± 233**	**0 ± 0**	**2 ± 5**	**4 ± 7**	**57 ± 11**

[a]Oncotic and necrotic OHCs were in and adjacent to focal lesions.

condensed into globular or crescent-shaped forms. Cells following the oncotic death pathway are swollen, pale-staining and have normal-sized to swollen nuclei [32-36]. Cochlear hair cells following the apoptotic and oncotic death pathways [24, 37, 38] have similar morphological characteristics to cells dying by these pathways in other tissues.

In addition to apoptosis and oncosis, several other death pathways have been identified in specific cell types [32, 34-36, 39-41]. According to Melino *et al.* [35], there are at least 11 morphologically distinct cell death pathways in different tissues. In the organ of Corti, Bohne *et al.* [24] described a possible new death pathway in noise-damaged OHCs that was morphologically distinct from apoptosis and oncosis (i.e., non-apoptotic and non-oncotic). OHCs that are dying by this latter death pathway have cellular debris (i.e., predominantly small, clear vacuoles) arranged in the shape of an intact OHC and a normal-sized or slightly swollen nucleus that is deficient in nucleoplasm but is positioned near the cell's base, as in a normal OHC. The use of *in vivo* trypan-blue staining showed that OHCs following the non-apoptotic, non-oncotic death pathway have leaky (i.e., disrupted) basolateral plasma membranes whereas apoptotic and oncotic OHCs have intact basolateral plasma membranes (Table **1**).

As discussed in Bohne *et al.* [24], it is unlikely that the death pathway identified here as non-apoptotic and non-oncotic represents a variant of apoptosis or oncosis. Each of these three death pathways is morphological distinct. OHCs following each of these death pathways were found simultaneously in the same region of individual cochleae. Bohne *et*

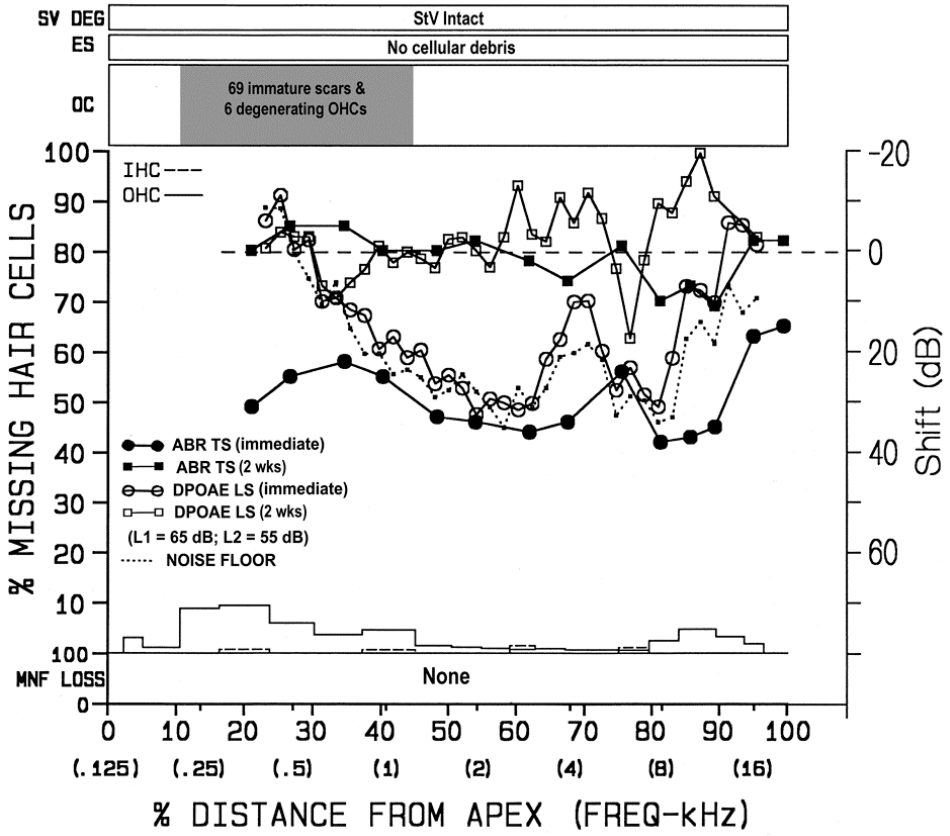

Fig. (8). Cytocochleogram for #1017R with functional overlay. At < 3-h post-exposure, ear had ABR TS (solid circles) of 15-38 dB from 0.375-20 kHz and DPOAE LS (open circles) of 10-33 dB from 0.6-8.8 kHz compared to pre-exposure data (horizontal dashed line). Dotted line represents differential noise floor for DPOAE LSs. By 2 wk post-exposure, function had completely recovered (i.e., within 10 dB pre-exposure), except for ABR TS of 11 dB at 12 kHz (solid squares) and DPOAE LS of 17 dB at 6.4 kHz (open squares). There were 4.9% missing OHCs in apical OC and no basal focal lesion. Number and location of degenerating OHCs and immature phalangeal scars are indicated in gray area of OC box.

al. [24] also showed that the non-apoptotic, non-oncotic death pathway occurs in gerbil OHCs. Inspection of published TEMs of noise-exposed cochleae reveals that hair cells which appeared to be following a non-apoptotic, non-oncotic death pathway have been seen previously in guinea pigs [6, 42, 43] and chinchillas [44].

One death pathway that appears morphologically similar to the non-apoptotic, non-oncotic death pathway described here for OHCs was termed 'paraptosis' [40]. Using cultured human embryonic kidney 293T cells and mouse embryonic fibroblasts, Sperandio *et al.* [40] noted that cells dying by paraptosis had extensive clear vacuoles in their cytoplasm, mitochondrial swelling and sometimes chromatin condensation, but no autophagic vacuoles and no apoptotic body formation. In addition, these cells were trypan-blue positive. Additional studies must be done on the non-apoptotic, non-oncotic death pathway in noise-damaged OHCs to determine if they exhibit other characteristics of paraptosis.

In the < 3-h recovery cochleae in the present study, many of the missing OHCs that were replaced by immature phalangeal scars and most of the OHCs that were degenerating were following a non-apoptotic, non-oncotic death pathway (Table **3**). The number of OHCs that died by the non-apoptotic, non-oncotic death pathway was statistically greater than the number of OHCs that died by either oncosis or apoptosis. Furthermore, the number of OHCs that died *via* the non-apoptotic, non-oncotic death pathway in the apical OC was significantly greater compared to the number in the basal OC. Our results are in contrast to those of Hu and colleagues [37, 38, 45-48] and Han *et al.* [49] where noise-damaged OHCs were reported to die predominantly *via* apoptosis. Differences in cochlear fixation, preparation and evaluation techniques, criteria for identifying dying cells and the higher noise levels, including impulses, used to damage the cochlea may account for the discrepancy between our results and those published previously.

All non-exposed control cochleae have some OHCs that died by an unknown death pathway (i.e., replaced by mature phalangeal scars). The timing of degeneration for these OHCs cannot be determined. Because of the 24-h exposure and the short recovery, there was insufficient time in the < 3-h recovery cochleae for OHCs to be damaged by the noise, to degenerate and for mature phalangeal scars to form. Thus, in these cochleae, OHCs dying by an unknown death pathway represent cell loss that preceded the noise exposure. The number of cells dying by an unknown death pathway was fairly similar among controls and no-recovery cochleae (Table **3**). In the 1- and 2-wk recovery cochleae, the number of cells dying by an unknown death pathway was increased

Table 4. Summary of ABR Threshold Shifts and DPOAE Level Shifts in Noise-Exposed Chinchillas

Ear # / Recovery	ABR TS[a] (dB) at [Freq (kHz)]	Max TS (dB) at [Freq (kHz)]	DPOAE LS[b] (dB) at [Freq[c] (kHz)]	Max LS (dB) at [Freq[c] (kHz)]
1016L (< 3 h)	45 [0.4] 15 [20]	66 [4]	14 [0.5] 14 [13.3]	36 [7.5]
1016R (< 3 h)	46 [0.4] 25 [20]	60 [4]	17 [0.5] 29 [12]	37 [7.9]
1022L (< 3 h)	25 [0.4] 25 [20]	63 [4]	12 [0.3] 23 [14.8]	35 [3.1]
1022R (< 3 h)	20 [0.4] 25 [20]	64 [6]	14 [0.3] 17 [14.8]	47 [4.2]
1017L[d] (< 3 h)	41 [0.4] 25 [20]	61 [4]	21 [0.5] 20 [14.8]	48 [4.2] 46 [8.8]
1017R[d] (< 3 h)	31 [0.4] 15 [20]	38 [8]	10 [0.6] 21 [8.8]	33 [2.0] 31 [7.9]
1018L[d] (< 3 h)	47 [0.4] 23 [20]	65 [3]	17 [0.9] 17 [14.8]	59 [6.4]
1018R[d] (< 3 h)	40 [0.4] 22 [20]	61 [3]	11 [0.4] 18 [13.3]	53 [7.1]
1024R[d] (< 3 h)	27 [0.4] 20 [20]	41 [8]	13 [1.5] 10 [14.8]	61 [5.2]
Mean (S.D.)[e]	**40 (5) [0.4 (-)] 22 (3) [20 (-)]**	**61 (4) [4.3 (1.1)]**	**15 (3) [0.6 (0.1)] 20 (5) [13.5 (2.2)]**	**51 (7) [4.4 (1.5)]**
1017L[f] (2 wk)	10 [6] 10 [16]	12 [10]	10 [3.1] 12 [12.0]	18 [4.2]
1017R[f] (2 wk)	10 [8] 11 [12]	11 [12]	17 [6.4]	17 [6.4]
1018L[f] (2 wk)	No shift	No shift	No shift	No shift
1018R[f] (2 wk)	No shift	No shift	13 [7.9]	13 [7.9]
1024R[f] (1 wk)	10 [8] 12 [10]	12 [10]	No shift	No shift

[a]Auditory brainstem response (ABR) threshold shift (TS) ≥ 10 dB.
[b]Distortion product otoacoustic emission (DPOAE) level shift (LS) ≥ 10 dB.
[c]For f_1.
[d]Determined immediately post-exposure in 1- or 2-wk recovery animals.
[e]Logarithmic mean shift and S.D.; harmonic mean frequency and S.D.
[f]Group means and S.D.s not calculated due to null data entries.

over those in the controls and < 3-h recovery cochleae. The logical explanation for this increase is that some of the OHCs that degenerated as a result of the exposure had had sufficient time to be replaced by mature phalangeal scars.

Trypan-Blue Protocol to Identify Hair Cells with Disrupted Plasma Membranes

The percentages of missing hair cells in controls instilled with trypan blue were similar to those in cochleae not treated with trypan blue, including our historical laboratory controls [11]. Thus, it was concluded that neither trypan blue nor the surgery associated with *in vivo* instillation of trypan blue into scala tympani had any significant deleterious effect on the OC. The presence of a few immature scars and degenerating OHCs in the controls probably indicates that cochlear surgery (i.e., opening of the bulla, manipulation of the ossicular chain, drilling perfusion holes in the perilymphatic spaces) causes a minimal amount of damage in the OC.

We found that the best protocol for identifying degenerating hair cells included the following steps: 1) *In vivo* instillation of 1% trypan blue in HBSS in scala tympani to avoid post-mortem artifacts, including the post-mortem injury and death of hair cells; 2) Instillation of sufficient dye into scala tympani to stain all degenerating cells in the OC. The trypan-blue solution was instilled into an inlet hole in scala tympani at the base until it escaped from an outlet hole near the apex. The trypan-blue solution was allowed to remain in scala tympani for 15 min; 3) Removal of unbound dye from scala tympani by perfusion with HBSS prior to cochlear fixation. This step was necessary to avoid staining healthy cells during fixation when the plasma membranes of all cells are rendered semi-permeable; 4) Primary fixation for 8 min with 1% OsO_4 in potassium dichromate buffer with 1.65% $CaCl_2$. This fixation protocol did not mask trypan-blue staining of cells with disrupted basolateral plasma membranes but it did allow visualization of all parts of the hair cells so that cell-death pathways could be determined by morphological criteria.

Pappenheimer [50] was first to use trypan blue for analyzing cell death. This method of assessing cell viability is based on the principle that certain dyes are excluded from penetrating into living cells with intact plasma membranes. Cells with intact plasma membranes exclude the dye while the nuclei in cells with disrupted plasma membranes stain with the dye. This test is sometimes termed the trypan-blue-exclusion test or assay.

Yamane *et al.* [51] and Sunami *et al.* [52] were first to apply the trypan-blue exclusion test to identify cell death in the OC. Their method involved sacrificing the animal, excising its cochleae and immersing them in a solution of trypan blue for 5 min. The cochleae were subsequently fixed in a buffered solution of 2.5% glutaraldehyde. The trypan-blue solution was not washed out of the cochlear scalae before the cochleae were fixed. Their *in vitro* protocol has shortcomings. First, cochlear immersion is an unreliable means of introducing trypan blue into the cochlea, especially for short treatment times. Second, because exposure to trypan blue took place after the animals' deaths, some of the stained cells could have been damaged or died shortly after sacrifice. Third, fixation of the cochlea before removal of unbound trypan blue results in indiscriminate staining of cells in the OC.

In our cochleae, the cells with disrupted plasma membranes contained trypan-blue-stained nuclei. Phase-contrast microscopic examination of degenerating OHCs with blue-stained nuclei revealed that they were either necrotic or were following a non-apoptotic, non-oncotic death pathway. OHCs that were apoptotic or oncotic did not stain with trypan blue. Thus, instillation of trypan blue in scala tympani allowed us to distinguish among hair cells with intact basolateral plasma membranes and those with disrupted basolateral membranes, provided that the dye was washed out of the cochlea prior to fixation.

Quantifying Missing and Degenerating Hair Cells

In order to examine the control and experimental cochleae for positive trypan-blue staining, each specimen was embedded in plastic while most of the cochlear bone was intact [23]. After the plastic polymerized, the cochleae were dissected so that flat preparations of the entire OC could be obtained. This technique avoided dissection artifacts in the delicate OC and permitted quantitative analysis of all hair cells from apex to base. Our analysis permitted a consistent, non-biased quantification of all degenerating cells whereas other studies usually evaluated samples of the OC only [37, 49, 52]. Our type of analysis is especially important when focal lesions may be present in the OC.

Variability in the Number of Trypan-Blue-Stained OHCs

In the nine cochleae in the < 3-h recovery group, there were a variable number of trypan-blue-stained OHC nuclei in the apical OC. This variability suggests that in different animals, OHCs were degenerating and/or nuclear debris was being cleaned up at different rates. In these same cochleae, only a few OHCs were apoptotic or oncotic and neither apoptotic nor oncotic cells contained blue-stained nuclei. In the basal OC, two of nine cochleae had focal OHC lesions. These lesions contained only a small number of nuclei stained with trypan blue. This finding indicates that these basal focal lesions occurred early during the exposure and/or nuclear debris from the degenerated OHCs in the base was cleaned up more rapidly than that from the apex.

In the five cochleae with the 1 or 2 wk of recovery, there were fewer trypan-blue-stained OHC nuclei in the apical OC than in the < 3-h recovery cochleae. This finding suggests that most apical hair cells were lost during the exposure and that substantial degeneration in the apex did not continue post-exposure. This finding supports the conclusion of Harding and Bohne [53] that with the 0.5-kHz OBN, OHCs generally die during the exposure. In the basal OC, three of these cochleae had focal lesions and one of the lesions contained some degenerating OHCs with trypan-blue-stained nuclei. This finding implies that following exposure to low-frequency noise, some basal hair-cell losses may occur post-exposure.

Structural and Functional Consequences of Exposure to the 0.5-kHz OBN

All noise-exposed cochleae sustained a variable amount of OHC loss scattered in the apical OC. Statistically, there was no difference in the amount of apical OHC loss between cochleae in the < 3-h group and those in the 1- or 2-wk recovery group. Focal losses of OHCs appeared sporadically in the basal OC in the < 3-h recovery cochleae. A larger percentage of the 1- or 2-wk recovery cochleae had basal focal lesions.

The magnitudes of OHC and IHC losses in the apical OC and basal OC in the < 3-h and 1- or 2-wk recovery cochleae were insufficient to account for the moderate-level, broadly distributed ABR threshold shifts and DPOAE level shifts that were measured immediately post-exposure. Likewise, there was also a poor correlation between hair-cell losses and residual ABR threshold shifts and DPOAE level shifts in the 1- and 2-wk recovery cochleae. These results are similar to those reported for the basal OC following exposure to a 4-kHz OBN [21, 54].

A variable number of oncotic OHCs were found in the OC bordering the basal focal lesions. None of these oncotic cells had blue-stained nuclei. Because of their very different morphological appearances, including not staining with trypan blue, we conclude that the mechanism of cell death for oncotic OHCs is different from that leading to death by a pathway that is morphologically distinct from apoptosis and oncosis.

Cellular Debris in the OC

In the 1- or 2-wk recovery cochleae, the losses of OHCs and IHCs were significantly greater than those in controls but not significantly different from those in the < 3-h recovery cochleae. Many of the degenerated OHCs had been replaced by mature phalangeal scars. There were generally no degenerating nuclei, no recognizable nuclear debris, or blue-stained nuclei in the OC. The lack of blue-stained nuclei suggests that nuclear debris from the degenerating OHCs was no longer stainable with trypan blue or had disappeared from the cochlea. If the debris disappeared, it may have broken up into small fragments that were removed by phagocytes during the 1-2 wk recovery period. It is also possible that small nuclear fragments may not have been recognizable by light microscopy. If nuclear material was truly absent from the apical half of the recovered cochleae, it has yet to be determined how and when the debris was removed from the OC.

In the present study, OHCs degenerating by the non-apoptotic, non-oncotic death pathway were identified morphologically. In addition, it was shown using *in vivo* trypan blue that these cells have disrupted basolateral plasma membranes whereas apoptotic and oncotic cells have intact plasma membranes. Further studies are needed to determine the molecular characteristics of OHCs degenerating by this alternate death pathway. However, techniques must be developed that allow molecular probes to be used with plastic-embedded tissue in which cell morphology is visible as well.

CONCLUSIONS

1. The best vital-dye protocol for identifying degenerating hair cells in the cochlea involved *in vivo* instillation of a 1% solution of trypan blue in HBSS in scala tympani for 15 min, followed by removal of unbound dye from the perilymphatic spaces then an 8-min fixation with a buffered solution of 1% osmium tetroxide. This protocol permitted the identification of degenerating cells with disrupted plasma membranes and other injured cells with intact plasma membranes.

2. Exposure to a 0.5-kHz OBN at 95 dB SPL for 24 h resulted in a consistent pattern of scattered loss of OHCs in the apical OC and an occasional focal (concentrated) loss of OHCs in the basal OC. In the apical OC of the < 3-h recovery cochleae, a variable number of degenerating OHCs were following a non-apoptotic, non-oncotic death pathway, while the numbers of apoptotic and oncotic OHCs was considerably less. Adjacent to the basal focal lesions, a variable number of oncotic OHCs were found. The different patterns of hair-cell loss at the apex and base following exposure to the low-frequency noise suggest that OHCs degenerating at the apex and base are dying by different mechanisms.

3. Degenerating OHCs following a non-apoptotic, non-oncotic death pathway could be observed with phase-contrast microscopy in flat preparations of the OC. These cells had cellular debris arranged in the typical shape of an OHC body, a disrupted basolateral plasma membrane and a nucleus located in its typical position but deficient in nucleoplasm. *In vivo* treatment with trypan blue resulted in the staining of the nuclei of these OHCs while those in apoptotic and oncotic OHCs did not stain.

ACKNOWLEDGEMENTS

This study was completed by the first author in partial fulfillment of the requirements for a MA in Biology and Biological Sciences at Washington University.

Support for this study was provided by the National Institute for Occupational Safety and Health (NIOSH), the Division of Biology and Biomedical Sciences, and the Department of Otolaryngology - Head and Neck Surgery at Washington University School of Medicine. The views expressed here are the sole responsibility of the authors and do not necessarily represent the views of NIOSH.

REFERENCES

[1] NIOSH (National Institute for Occupational Safety and Health). Work-Related Hearing Loss. Pub 2001-103.

[2] NIDCD (National Institute on Deafness and Other Communication Disorders). Noise-Induced Hearing Loss 2007; NIH Pub 97-4233.

[3] Spoendlin H. Primary structural changes in the organ of Corti after acoustic overstimulation. Acta Otolaryngol 1971; 71: 166-176.

[4] Spoendlin H. In: Henderson D, Hamernik RP, Mills JH, Dosanjh DS Eds, Anatomical Changes Following Various Noise Exposures. In Effects of Noise on Hearing. NY, Raven Press, 1976; 69-87.

[5] Bohne BA. In: Henderson D Hamernik RP, Mills JH, Dosanjh DS Eds, Mechanisms of Noise Damage in the Inner Ear. In Effects of Noise on Hearing. NY, Raven Press, 1976; 41-68.

[6] Lim DJ, Dunn DE. Anatomic correlates of noise induced hearing loss. Otolaryngol Clin N Amer 1979; 12: 493-513.

[7] Yamane H, Nakai Y, Takayama M, *et al*. The emergence of free radicals after acoustic trauma and strial blood flow. Acta Otolaryngol 1995; (Suppl 519) 87-92.

[8] Ohlemiller KK, Wright JS, Dugan LL. Early elevation of cochlear reactive oxygen species following noise exposure. Audiol Neurootol 1999; 4: 229-236.

[9] Henderson D, Bielefeld EC, Harris KC, Hu BH. The role of oxidative stress in noise-induced hearing loss. Ear Hear 2006; 27: 1-19.

[10] Slepecky NB. In: Dallos P, Popper AN, Fay RR Eds, Structure of the Mammalian Cochlea. In The Cochlea. NY, Springer, 1996; 44-129.

[11] Bohne BA. Safe level for noise exposure? Ann Otol Rhinol Laryngol 1976; 85: 711-724.

[12] Fried MP, Dudek SE, Bohne BA. Basal turn cochlear lesions following exposure to low-frequency noise. Trans Amer Acad Ophthalmol Otolaryngol 1976; 82: 285-298.

[13] Bohne BA, Clark WW. In: Hamernik RP, Henderson D, Salvi R Eds, Growth of Hearing Loss and Cochlear Lesion with Increasing Duration of Noise Exposure. In New Perspectives on Noise-Induced Hearing Loss. NY, Raven Press, 1982; 283-302.

[14] Salvi R, Perry J, Hamernik RP, Henderson D. In: Hamernik RP, Henderson D, Salvi R Eds, Relationships Between Cochlear Pathologies and Auditory Nerve and Behavioral Responses Following Acoustic Trauma. In New Perspectives on Noise-Induced Hearing Loss. NY, Raven Press, 1982; 165-188.

[15] Harding GW, Bohne BA. Distribution of focal lesions in the chinchilla organ of Corti following exposure to a 4-kHz or a 0.5-kHz octave band of noise. Hear Res 2007; 225: 50-59.

[16] Bohne BA, Harding GW, Nordmann AS, Tseng CJ, Liang GE, Bahadori RS. Survival-fixation of the cochlea: A technique for following time-dependent degeneration and repair in noise-exposed chinchillas. Hear Res 1999; 134: 163-178.

[17] Yamashita D, Jiang H, Le Prell C, Schacht J, Miller JM. Post-exposure treatment attenuates noise-induced hearing loss. Neuroscience 2005; 134: 633-642.

[18] Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 1995; 146: 3-15.

[19] Majno G, Joris I. Cells, Tissues and Disease. Principles of General Pathology. Cambridge, MA, Blackwell Science 1996; 190-221.

[20] Jugdutt BI, Idikio HA. Apoptosis and oncosis in acute coronary syndromes: Assessment and implications. Mol Cell Biochem 2005; 270: 177-200.

[21] Nordmann AS, Bohne BA, Harding GW. Histopathological differences between temporary and permanent threshold shift. Hear Res 2000; 139: 13-30.

[22] Harding GW, Bohne BA, Ahmad M. DPOAE level shifts and ABR threshold shifts compared to detailed analysis of histopathological damage from noise. Hear Res 2002; 174: 158-171.

[23] Bohne BA, Harding GW. Combined organ of Corti/modiolus technique for preparing mammalian cochleas for quantitative microscopy. Hear Res 1993; 71: 114-124.

[24] Bohne BA, Harding GW, Lee SC. Death pathways in noise-damaged outer hair cells. Hear Res 2007; 223: 61-70.

[25] Bohne BA. In: Hirsh SK, Eldredge DH, Hirsh IJ, Silverman SR Eds, Healing of the Noise-Damaged Inner Ear. In Hearing and Davis: Essays Honoring Hallowell Davis. St. Louis, Washington University Press, 1976; 85-96.

[26] Bohne BA, Gruner MM, Harding GW. Morphological correlates of aging in the chinchilla cochlea. Hear Res 1990; 148: 79-92.

[27] Bohne BA, Bozzay DG, Harding GW. Interaural correlations in normal and traumatized cochleas: Length and sensory cell loss. J Acoust Soc Am 1986; 80: 1729-1736.

[28] Bohne BA, Carr CD. Location of structurally similar areas in chinchilla cochleas of different lengths. J Acoust Soc Am 1979; 66: 411-414.

[29] Bohne BA, Carr CD. Morphometric analysis of hair cells in the chinchilla cochlea. J Acoust Soc Am 1985; 77: 153-158.

[30] Bohne BA, Harding GW. Degeneration in the cochlea after noise damage: Primary versus secondary events. Am J Otol 2000; 21: 505-509.

[31] Eldredge DH, Miller JD, Bohne BA. A frequency-position map for the chinchilla cochlea. J Acoust Soc Am 1981; 69: 1091-1095.

[32] Blank M, Shiloh Y. Programs for cell death. Apoptosis is only one way to go. Cell Cycle 2007; 6: 686-695.

[33] Bröker LE, Kruyt FAE, Giaccone G. Cell death independent of caspases: A review. Clin Cancer Res 2005; 11: 3155-3162.

[34] Golstein P, Kroemer G. A multiplicity of cell death pathways. Symposium on apoptotic and non-apoptotic cell death pathways. EMBO Rep 2007; 8: 829-833.

[35] Melino G, Knight RA, Nicotera P. How many ways to die? How many different models of cell death? Cell Death Differ 2005; 12: 1457-1462.

[36] Wyllie AH, Golstein P. More than one way to go. Proc Natl Acad Sci 2001; 98: 11-13.

[37] Hu BH, Guo W, Wang PY, Henderson D, Jiang SC. Intense noise-induced apoptosis in hair cells of the guinea pig cochleae. Acta Otolaryngol 2000; 120: 19-24.

[38] Yang WP, Henderson D, Hu BH, Nicotera TM. Quantitative analysis of apoptotic and necrotic outer hair cells after exposure to different levels of continuous noise. Hear Res 2004; 196: 69-76.

[39] Latour I, Leunda-Casi A, Denef J-F, Calderon PB. Activation of poly(ADP-ribose) polymerase in rat hepatocytes does not contribute to their cell death by oxidative stress. Exp Cell Res 2000; 254: 173-197.

[40] Sperandio S, de Belle I, Bredesen DE. Alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci 2000; 97: 14376-14381.

[41] Taper HS, Jamison JM, Gilloteaux J, Gwin CA, Gordon T, Summer JL. *In vivo* reactivation of DNases in implanted human prostate tumors after administration of a vitamin C/K_3 combination. J Histochem Cytochem 2001; 49: 109-119.

[42] Spoendlin H In: Babel J, Bischoff A, Spoendlin H. Eds, Auditory, vestibular, olfactory and gustatory organs. In Ultrastructure of the Peripheral Nervous System and Sense Organs. Atlas of Normal and Pathologic Anatomy. St Louis, MO, CV Mosby Co, 1970; 240-241.

[43] Fredelius L. Time sequence of degeneration pattern of the organ of Corti after acoustic overstimulation. A transmission electron microscopic study. Acta Otolaryngol 1988; 106: 373-385.

[44] Ward WD, Duvall AJ III. Behavioral and ultrastructural correlates of acoustic trauma. Ann Otol Rhinol Laryngol 1971; 80: 881-896.

[45] Hu BH, Henderson D, Nicotera TM. Involvement of apoptosis in progression of cochlear lesion following exposure to intense noise. Hear Res 2002a; 166: 62-71.

[46] Hu BH, Henderson D, Nicotera TM. F-actin cleavage in apoptotic outer hair cells in chinchilla cochleas exposed to intense noise. Hear Res 2002b; 172: 1-9.

[47] Hu BH, Henderson D, Nicotera TM. Extremely rapid induction of outer hair cell apoptosis in the chinchilla cochlea following exposure to impulse noise. Hear Res 2006; 211: 16-25.

[48] Nicotera TM, Hu BH, Henderson D. The caspase pathway in noise-induced apoptosis of the chinchilla cochlea. J Assoc Res Otolaryngol 2003; 4: 466-477.

[49] Han W, Shi X, Nuttall AL. AIF and endoG translocation in noise exposure induced hair cell death. Hear Res 2006; 211: 85-95.

[50] Pappenheimer AJ. Experimental studies upon lymphocytes: The reactions of lymphocytes under various experimental conditions. J Exp Med 1917; 25: 633-650.

[51] Yamane H, Konishi K, Iguchi H, *et al*. Assessment of hair cell death using the dye extrusion method. Acta Otolaryngol 1998; (Suppl 538) 7-11.

[52] Sunami K, Yamane H, Takayama M, Nakagawa T, Konishi K, Iguchi H. Cystine protects cochlear outer hair cells against glutamate toxicity. Acta Otolaryngol 1999; 199: 671-673.

[53] Harding GW, Bohne BA. Noise-induced hair-cell loss and total energy: Analysis of a large data set. J Acoust Soc Am 2004; 115: 2207-2220.

[54] Harding GW, Bohne BA. Temporary DPOAE level shifts, ABR threshold shifts and histopathological damage following below-critical-level noise exposures. Hear Res 2004; 196: 94-108.

Received: February 26, 2008 Revised: March 3, 2008 Accepted: March 6, 2008