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COMPARATIVE ANALYSIS OF PATCH LESIONS IN THE CHICK INNER EAR FOLLOWING ACOUSTIC TRAUMA: OPTICAL VERSUS SCANNING ELECTRON MICROSCOPY

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Abstract

Neonatal chicks were exposed to an octave band noise with a center frequency of 1.5 kHz at 116 dB SPL for 4 hours. Seven days following overstimulation, the birds were sacrificed. Their basilar papillae were removed, fixed in 4% paraformaldehyde, and processed in two steps. First, the ears were immunostained with a supernatant of mouse anti-tectorial membrane antibodies, followed by a diaminobenzidine process. Examinations of the papillae under an optical stereo microscope revealed a patch site with a partially regenerated tectorial membrane (referred to as the honeycomb).

After the optical studies, the same ears were post-fixed in 1% osmium tetroxide, dehydrated in ethanol, and processed for scanning electron microscopy (SEM). SEM examinations demonstrated a honeycomb-covered patch lesion in the papilla. Patch lesion perimeters were traced from both the optical and SEM images, and patch areas were calculated. Also, papilla height was measured at the midpoint of the inner ear in both groups. These calculations showed that the patch area and papilla height had shrunk by approximately 37% and 33%, respectively, following the SEM methodology. The decrease in these dimensions may be attributed to several steps required for the SEM specimen preparation, such as critical point drying.

Key words: Chick, basilar papilla, tectorial membrane, acoustic overstimulation, regeneration, immunocytochemistry.

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Introduction

Many structural changes in the avian basilar papilla following acoustic overstimulation are found in a "patch" lesion located at a papilla region most sensitive to the exposure frequency. This site of injury displayed distorted apical surfaces of hair cells and supporting cells, hair cell loss, and tectorial membrane destruction. Within one week following overstimulation, the cell apical surfaces returned to near normal, nearly all lost hair cells were replaced with new ones, and a honeycomb-like layer overlay the patch lesion (see for review: Rubel, 1992; Saunders *et al.*, 1992; Cotanche *et al.*, 1994).

In the present study, we used two different methods to analyze the patch lesion caused by intense sound exposure, one of these being scanning electron microscopy (SEM). SEM has been instrumental in elucidating many important elements of papilla damage and repair (see for review: Rubel, 1992; Saunders *et al.*, 1992; Cotanche *et al.*, 1994), and also has helped determine differences in inner ear injury following overexposure, using various parameters. For example, exposure of increasing intensity or at an older age causes a larger lesion as well as a greater degree of papilla damage (Cotanche *et al.*, 1987; Adler *et al.*, 1992, 1993; Adler and Saunders, 1995). However, the quantitative differences in papilla damage may be underestimated, since specimen preparation for SEM involves many steps, which may cause severe changes in the cellular structures in the ear. These steps include buffer storage, aldehyde fixation, osmium post-fixation, alcohol dehydration, and critical point drying (Schneider, 1976; Boyde *et al.*, 1977; Boyde and Maconnachie, 1979, 1981).

An alternative approach to SEM is to visualize whole-mounts of the basilar papilla at the light microscopic level after histo- or immuno-chemical labeling procedures (Raphael, 1991, 1992, 1993). We have recently used this immunostaining technique with monoclonal antibodies against the avian tectorial membrane (Adler *et al.*, 1995). This method helped locate the patch lesion, and determine that both chick and quail inner ears are able to partially regenerate their tectorial

membrane following acoustic trauma (Adler *et al.*, 1995). Since the immunocytochemical procedure eliminates the use of osmium tetroxide, alcohol, and critical point drying, we hypothesize that the structural dimensions of bird basilar papillae following the labeling method are different from those obtained from SEM. Specifically, in the present study, we compared immunocytochemical and SEM observations of the ears one week after acoustic overstimulation. Patch lesion area and basilar papilla height were measured in order to quantitatively determine the structural differences in specimens prepared for and observed by the two techniques.

Materials and Methods

Animals

Chicks (*Gallus domesticus*, 7-14 days of age) were exposed to an octave band noise with a center frequency of 1.5 kHz at 116 dB SPL for 4 hours.

Immunocytochemistry

The immunolabeling method has been described elsewhere (Adler *et al.*, 1995). Briefly, one week after overstimulation, the birds were anesthetized with 35% chloral hydrate, and their temporal bones removed. The basilar papillae were exposed and fixed in 4% paraformaldehyde (pH 7.3) for 2 hours. Following the removal of surrounding tissue including the tegmentum vasculosum, the ears were permeabilized in 0.3% Triton X-100 for 10 minutes. After several phosphate buffered saline (PBS; pH 7.3) washes, the papillae were incubated overnight in an undiluted supernatant of mouse anti-tectorial membrane antibodies (identified as "TM-1;" Goodyear *et al.*, 1994) at room temperature. The papillae were washed in PBS, incubated in a 1:200 solution of biotinylated horse anti-mouse antibodies for 30 minutes, rinsed again in PBS, immersed in an A + B complex solution (Vectastain, Vector Labs., Burlingame, CA) for 30 minutes, washed in PBS, and finally cross-reacted with a diaminobenzidine-H_2O_2 solution for 10 minutes. The ears were placed in whole mounts, and examined under an optical stereo microscope (Nikon SMZ-U Zoom 1:10). Photographs were taken of these papillae at 75x. Throughout the text, the stereo microscopic examinations are referred to as "optical."

Scanning electron microscopy (SEM)

Following the optical examinations, the papillae were fixed in 1% OsO_4 (pH 7.3) for 45 minutes, and dehydrated in increasing concentrations of ethanol (35, 50, 70, 80, 90, 95, and 3 times in 100%) for ten minutes each. Finally, the specimens were critical point-dried in CO_2. The ears were then sputter-coated with gold/palladium, and examined in an AMRAY 1000 scanning electron microscope. Photomicrographs were taken at 200x.

Patch area and basilar papilla height analysis

The images scanned from photographs obtained during both the optical and SEM studies were analyzed, using MetaMorph (Universal Imaging Corporation, West Chester, PA). Patch lesion perimeters in all examined ears were traced, and patch areas calculated. At the approximate midpoint between the base and apex, basilar papilla height was measured, following a trace from superior to inferior papilla edge.

The data obtained from the optical examinations were compared with those from the SEM analysis, and independent *t*-tests were performed. Any probability of chance occurrences less than 5 out of 100 indicated reliable differences.

Results

Optical stereo microscopic observations

Figure 1A shows a chick inner ear 7 days after overstimulation. The crescent-shaped papilla was covered by a dark staining of the tectorial membrane. The patch lesion displayed a darkly stained honeycomb-like layer (Fig. 1A). A light background labeling was detected underneath the honeycomb at the level of the reticular lamina (Fig. 1A).

Scanning electron microscopic observations

The scanning electron micrographs of chick inner ears displayed improved resolution and, more importantly, greater detail than the stereo micrographs of the same papillae. As can be seen in Figure 1B, the papilla demonstrated a honeycomb covering the patch lesion. This observation is similar to that in Figure 1A. However, the SEM showed that the honeycomb consisted of a series of rings, or chalices, several of which could be observed in close proximity with hair cell stereocilia (Fig. 1B, inset). This detail could not be detected under a stereo microscope following the immunolabeling method with TM-1. Further descriptions of the honeycomb can be found elsewhere (Cotanche, 1987; Adler *et al.*, 1993; for general review on ear repair see: Rubel, 1992; Saunders *et al.*, 1992; Cotanche *et al.*, 1994).

Patch area and basilar papilla height analysis

Measurements of basilar papilla height and patch lesion area were obtained from both optical and SEM studies. The mean papilla heights in the optical and SEM samples are listed in Table 1. An independent *t*-test yielded reliable differences between the SEM and optical groups ($t = -9.03$, $df = 16$, $p < 0.0001$), and the shrinkage in height caused by the SEM preparation steps was 33%.

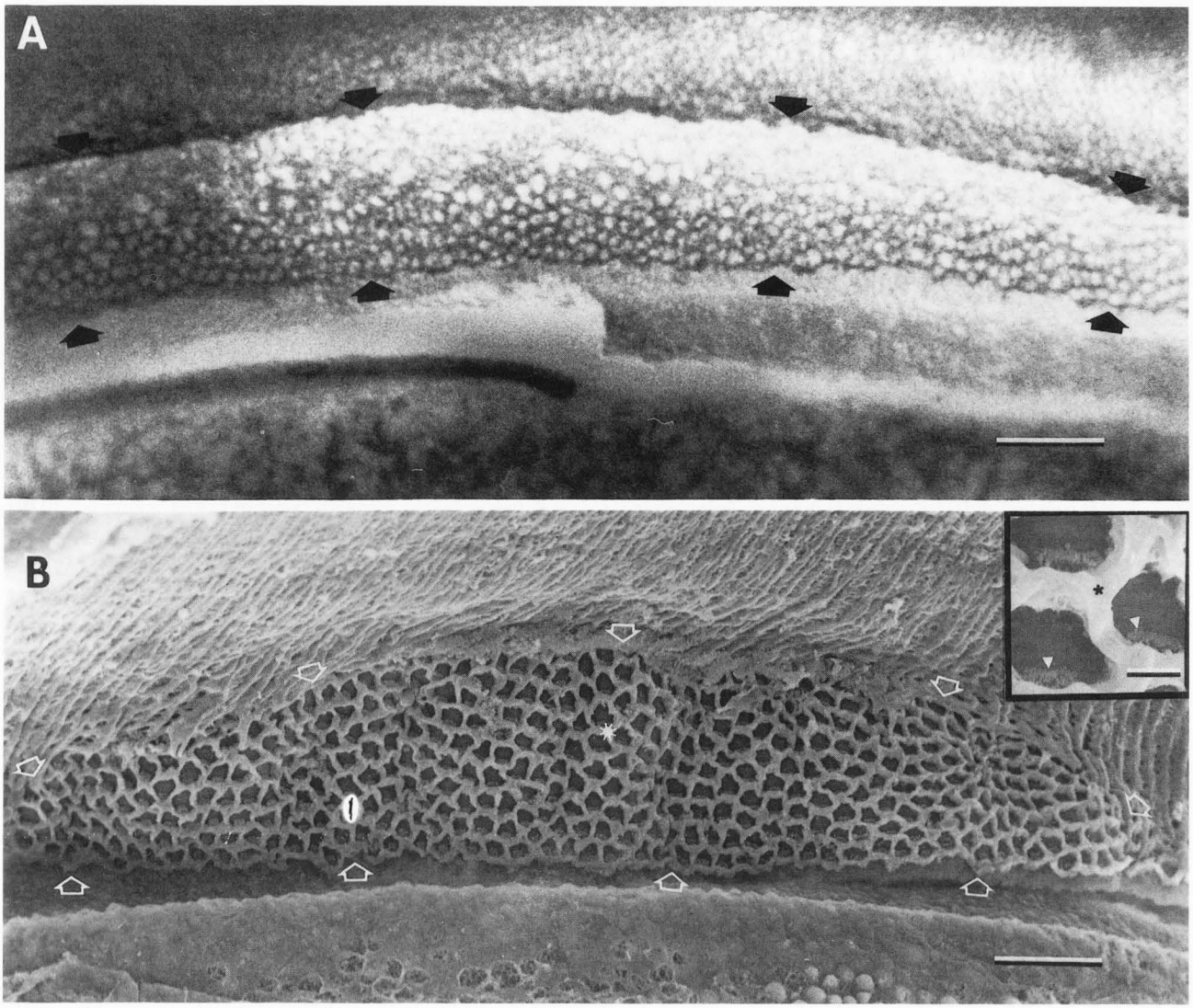

Figure 1A. A chick basilar papilla was viewed under an optical stereo microscope 7 days after exposure. A partially regenerated tectorial membrane, called the "honeycomb," is seen covering the patch lesion. The superior edge of the honeycomb is surrounded by the remnant of the original tectorial membrane. Solid arrows situate the borders of the patch. Space limitations caused the removal of the proximal and basal ends of the lesion from the photomicrograph.

Figure 1B. The chick papilla was then examined under SEM. This ear demonstrates the honeycomb, as indicated by hollow arrows. The white asterisk indicates the approximate location of the inset. **Inset**: Hair cell stereocilia (arrowheads) are in proximity with the chalices of the honeycomb (black asterisk). For Figures 1A and 1B, bars = 100 μm; for inset, bar = 5 μm.

Mean patch lesion areas also differed in the SEM and optical specimens and are listed in Table 2. An independent *t*-test revealed that the area differences were significant (t = -2.29, df = 16, $p < 0.05$), and indicated that the patch area shrank by 37% following SEM.

Discussion

Structural aspects of inner ear repair in acoustically injured birds have been well documented (for reviews, see: Rubel, 1992; Saunders *et al.*, 1992; Cotanche *et al.*, 1994). The present observations of the honeycomb at one week post-exposure merely confirmed one of the aspects, tectorial membrane regeneration. However, the major finding of the present investigation is that the processes between the times of the immunocytochemical and SEM analyses caused a large degree of shrinkage in two dimensions of the inner ear (papilla height and patch

Table 1. Basilar papilla height.

Method	Average Height[a]	S.E.[b]
Stereo Microscopy[c]	324	7.6
SEM[c]	216	9.3

[a]measured in μm. [b]S.E. = standard error.
[c]n = 9

Table 2. Patch lesion area.

Method	Patch Area[a]	S.E.[b]
Stereo Microscopy[c]	106,500	15,000
SEM[c]	67,000	8,400

[a]measured in μm^2. [b]S.E. = standard error
[c]n = 9

lesion area). The present amounts of shrinkage (33%-37%) were similar to those reported in earlier studies on different tissues and cells during SEM preparation. For example, several investigators reported a 30-45% reduction in the mean cellular diameter of isolated cells (Schneider, 1976; Billings-Gagliardi *et al.*, 1978; Schneider *et al.*, 1978). We, however, do not claim that on the basis of the shrinkage in patch area and papilla height, the immunocytochemical method provides a superior approach to analyze the structural organization of the papilla or any other tissue than SEM. This is in part because the scanning electron micrographs of the ear (Fig. 1B) demonstrated more detail than the immunocytochemical images of the same papilla (Fig. 1A). We only point out that the shrinkage is one risk associated with SEM, not the labeling method. However, fluorescent immunocytochemistry following co-localization of two or more antibodies may demonstrate more detail in the papilla (Raphael, 1992, 1993). It would be interesting to analyze the changes in the structural dimensions of hair cells and supporting cells following SEM and immunocytochemistry.

The contributions of SEM preparation methods (such as alcohol dehydration, osmium tetroxide fixation, and critical point drying) in the dimensional changes in several tissues have been discussed elsewhere. Many investigators indicated that most of the shrinkage was due to critical point drying, and attributed this effect to a combination of liquid loss within the affected tissues and pressure imposed on these tissues by critical point drying (Schneider, 1976; Boyde *et al.*, 1977; Billings-Gagliardi *et al.*, 1978; Schneider *et al.*, 1978; Boyde and Maconnachie, 1979; 1981).

The present study focused only on osmication, alcohol dehydration and critical point drying, because formaldehyde fixation preceded both the immunostaining and SEM techniques. Nevertheless, the effects of formaldehyde fixation on the ear structures must be considered. Several studies have shown that aldehydes (glutaraldehyde and formaldehyde) reduced cellular dimensions (Penttila *et al.*, 1974; Dam, 1979; Boonstra *et al.*, 1983). In addition, the general consensus for the SEM preparation calls for specimens to be fixed in glutaraldehyde prior to osmium post-fixation (Schneider, 1976; Boyde *et al.*, 1977; Schneider *et al.*, 1978; Billings-Gagliardi *et al.*, 1978; Boyde and Maconnachie, 1979, 1981). However, in the present study, the immunostaining method precluded the use of glutaraldehyde, yet the surface of the basilar papilla appeared well-preserved at the SEM level (Fig. 1B). It would be interesting to compare the patch area after paraformaldehyde or glutaraldehyde fixation, followed by SEM and whole-mount techniques.

It is important to note that the patch area was based on the perimeter of the partially regenerated tectorial membrane (the singularly layered honeycomb). The normal tectorial membrane is a gelatinous, bi- or tri-layered tissue, 97% of which consists of water (Tanaka and Smith, 1975; Cohen and Fermin, 1985; Thalmann *et al.*, 1987; Shiel and Cotanche, 1990; Killick *et al.*, 1992). Acoustic overstimulation produces a papilla lesion which lacks any tectorial membrane (Cotanche, 1987; Raphael, 1991; Adler *et al.*, 1993; W.C.P. Sheets and B.M. Ryals, 1994, personal communication). The mechanisms of tectorial membrane disintegration during noise remain unknown, but most of the observations on the tectorial membrane following noise were obtained via SEM (Cotanche, 1987; Raphael, 1991; Adler *et al.*, 1993; Sheets and Ryals, personal communication). This raises a serious concern that SEM preparation methods, not noise, may cause an injury in the tectorial membrane, because the tectorial membrane contains mostly water, which would be extracted by the SEM preparation steps. Fortunately, Cotanche (1992) used differential interference contrast (DIC) videomicroscopy, instead of SEM, to eliminate the potential problem due to the damage that SEM preparation could have inflicted on the tectorial membrane. Nonetheless, it has been shown that varying concentrations of sodium, potassium and/or calcium ions caused partially reversible changes in the size, shape and thickness of the normal chick tectorial membrane (Freeman *et al.*, 1994). Obviously, various fixa-

tives, buffers, and alcohol as well as critical point drying replaced water in the tectorial membrane, thus, altering its structures. These structural changes may partially explain why the region of tectorial membrane destruction is larger than that of hair cell damage and loss (Raphael, 1991). The changes in the tectorial membrane following immunocytochemical and SEM preparation suggest caution in drawing quantitative conclusions concerning patch area and basilar papilla height from scanning electron micrographs.

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Discussion with Reviewers

R. Wroblewski: Is it possible that alcohol dehydration which you have been using was not good enough to withdraw all the water from such a hydrous structure as the tectorial membrane? Do you have any results where alcohol dehydration was followed by more powerful acetone prior to critical point drying?

Authors: Yes, it is possible that alcohol may not remove all water (and ions in it) from the tectorial membrane, in part because alcohol itself may absorb different molecules (such as oxygen and water) from the room atmosphere, and these molecules may compromise alcoholš ability to dehydrate target tissues. However, we used fresh, not old, alcohol to reduce the likelihood for the alcohol to be affected by the room atmosphere.

Several investigations (Adler *et al.*, 1992; 1993) used acetone to dehydrate the acoustically damaged chick ear prior to SEM for the comparative studies following various exposure parameters. The average patch area in this papilla immediately and 12 days after exposure at 2-3 days of age ranged from 55,000 to 67,000 μm^2. The SEM data obtained in the present study fell within that range. The similar results following different dehydrating media suggest that the use of acetone as a dehydrator would give the same results as that of alcohol. However, the comparison between the present study and Adler *et al.*(1992, 1993)š observations needs to be viewed with caution, because the earlier investigations excluded any aldehyde fixation prior to osmium post-fixation and subsequent acetone dehydration.

R. Wroblewski: Have you been calibrating the scanning electron microscope against the stereo microscope using the same object?

Authors: We are not sure whether the reviewer meant by the ruler or by the same specimen. We used different rulers with same units (μm), and were able to correlate these rulers. We even were able to match the length of both the scale bars in Figures 1A and 1B, even though the stereo microscope had the maximum magnification of 75x.

As for calibrating both techniques with the same specimen, we had a great amount of difficulty in matching ears following the two methods for several reasons. First, the immunocytochemical technique with expensive antibodies required that the basilar papilla be freed of the surrounding tissue, that is, the cartilage that engulfs and supports the papilla. As a result of the loss of body support, both the apical and basal ends of the inner ear could not withstand the rigors of SEM preparation, i.e., ethanol dehydration and critical point drying. Second, SEM preparation may alter or eliminate any markers that could ease a match-up between the optical and SEM images.

M.L. Wiederhold: The finding of a reduced patch lesion area is somewhat surprising. Given the evidence that several SEM preparation procedures reduce cell size, one might expect the tectorial membrane to shrink even more, since fewer structural elements are present in the tectorial membrane, compared to cells. Was the total length of the basilar papilla measured before and after fixation? Did the patch area decrease to the same extent when expressed as a percentage of the total area or length of the basilar papilla, or does the reduction in patch area just represent overall preparation shrinkage?

Authors: We agree with your expectation that the tectorial membrane might shrink more, due to its simpler structural composition, than cells. However, we have to be cautious in comparing our studies with other investigations (e.g., Schneider, 1976; Billings-Gagliardi *et al.*, 1978; Schneider *et al.*, 1978) because the other studies targeted **isolated** cells, while we focused on the papilla region with the lesion. As you can see, there may be neighborly constraints provided by the underlying cells as well as the remaining tissues. These constraints may prevent the tectorial membrane from further shrinkage.

We were not able to examine the whole basilar papilla because, as we mentioned in our response to Dr. Wroblewskiš second question, SEM preparation altered the structures of the papilla, especially in the apical and basal ends. Thus, we were not able to measure the total length of the basilar papilla after fixation. Nonetheless, we were able to examine the patch lesion and to measure the papilla height at the approximate midpoint between

the apex and base. As mentioned in the text, the patch area decreased to nearly the same extent as the papilla height (37% in area versus 33% in height). In any case, it would be interesting to compare the area and/or length of the papilla before and after acoustic overstimulation.

The percentage decreases in the two dimensions represent overall preparation shrinkage because we did not examine any tissues prior to fixation. We had to fix the papillae immediately after sacrifice, because we feared that non-fixation following sacrifice may cause unnecessary changes in the papilla and its lesion.

M.L. Wiederhold: How do you know that there is no shrinkage (or swelling), relative to normal live dimensions, with fixation and staining?

Authors: As several investigations described (Penttila *et al.*, 1974; Dam, 1979; Boonstra *et al.*, 1983), aldehyde fixation caused a decrease in cellular dimensions. However, we do not know if antibody staining causes any changes in these structural dimensions, except that the antibody binds to whatever it is supposed to bind. Since DIC videomicroscopy does not require any fixation or labeling, Cotanche (1992) has successfully used this technique to demonstrate tectorial membrane injury and regeneration in the acoustically damaged chick ear. This technique has been used to show that the structural dimensions of the normal tectorial membrane were changed by varying concentrations of different ions such as sodium, potassium and calcium (Freeman *et al.*, 1994). It would be interesting to compare the dimensions of the normal and acoustically injured papillae during DIC videomicroscopic and immunocytochemical studies.

B. Canlon-Petersson: It is really not clear from either the stereo micrograph or the description in **Results** as to what, within the chick tectorial membrane, the mouse anti-tectorial membrane antibody is reacting with.

Authors: Although the purpose of the immunolabeling was to stain the chick tectorial membrane and its noise-induced changes, we have not yet determined exactly where the mouse anti-tectorial membrane antibody binds within the partially regenerated tissue. Nonetheless, we embedded several normal bird basilar papillae in plastic immediately after immunolabeling. These embedded specimens were thick-sectioned every 1 μm and stained with or without Toluidine blue. In either case, the brown staining was found on the surface of the honeycomb-patterned bottom layer of the tectorial membrane (data not shown; for further details, see Goodyear *et al.*, 1994).