Otology & Neurotology
34:353–359 © 2013, Otology & Neurotology, Inc.

''In-bone'' Utricle Cultures—A Simplified, Atraumatic Technique for In Situ Cultures of the Adult Mouse (*Mus musculus*) Utricle

*†§Henry C. Ou, *‡Vincent Lin, and *†Edwin W. Rubel

**Virginia Merrill Bloedel Hearing Research Center, †Department of Otolaryngology–Head and Neck Surgery, University of Washington, Seattle, Washington, U.S.A.; ‡Sunnybrook Health Sciences Centre, Department of Otolaryngology–Head and Neck Surgery, University of Toronto, Toronto, Ontario, Canada; and §Seattle Children's Hospital, Seattle, Washington, U.S.A.*

Hypothesis: The ''in-bone'' method of culturing utricles described here is a reliable and atraumatic technique for culturing mature mouse hair cells and studying hair cell death and protection.
Background: The current in vitro technique for studying hair cells of the mature mouse utricle involves removal from the temporal bone and free floating culture in media. This technique can be problematic because of variability in the preservation of the sensory epithelium and a steep learning curve that results in injury of the sensory epithelium in less experienced hands. We present a new atraumatic technique of culturing the utricle in situ within the temporal bone.
Methods: Leaving the temporal bone largely intact, a window is opened in the bony vestibule overlying the mouse utricle. The entire temporal bone is then placed into culture media. Utricles were cultured in situ for several days with minimal damage to the epithelium. The utricles are then fixed in situ, removed from the temporal bone, and processed. A standardized aminoglycoside-induced hair cell damage protocol was developed.
Results: Mature mouse utricles maintained hair cell numbers for 3 days in culture. Exposure to neomycin resulted in significant dose-dependent hair cell toxicity ($p < 0.0001$, 1-way analysis of variance). Exposure to the protective drug tacrine resulted in significant protection against neomycin ($p < 0.05$, 3-way analysis of variance).
Conclusion: The ''in-bone'' technique is a reliable and atraumatic method for culturing mature mouse utricles and studying hair cell death and protection. It is easily mastered and can make in vitro study of hair cells accessible to more research groups.
Key Words: Hair cells—Mouse—Utricle.

Otol Neurotol **34:**353–359, 2013.

Hair cell death and protection are frequently studied with in vitro preparations. In neonatal mice, this can be done with whole organ of Corti cultures, because of the improved survival of neonatal hair cells and ease of dissection. In contrast, mature mouse inner ear hair cells have been a challenge to maintain in culture. For this reason, free-floating vestibular macular cultures have been a useful tool for in vitro evaluation of the mature mouse hair cell. This technique has been used effectively in studies of hair cell regeneration and hair cell death, as well as various agents that protect hair cells (1–8).

The utility of the free-floating utricle preparation has been limited, however, by the technical difficulty of the dissection and the fragility of the fresh tissue when manipulations are required. Frequently, particularly in less experienced hands, there is mechanical damage to the sensory epithelium, leading to unusable tissue and more wastage of mice. The steep learning curve involved can make this technique cumbersome, particularly for new researchers seeking to gain experience with the inner ear, as well as students with less time to achieve technical mastery of a technique. In addition, it is inevitable that the process of avulsing the utricle from its neural connections may lead to intracellular events that predispose a hair cell to death, or otherwise alter the conditions of the tissue.

This report describes a simple atraumatic method for preparing mature mouse utricles for culture. The ''in-bone'' method was then used to directly compare neomycin-induced hair cell damage in this new preparation to the free-floating utricle method. Lastly, protection against neomycin-induced hair cell death by the protective drug tacrine was demonstrated using the in-bone method.

Address correspondence and reprint requests to Henry C. Ou, M.D., University of Washington, VMB Hearing Research Center, Box 357923, Seattle, WA 98195-7293; E-mail: henryou@u.washington.edu
The authors disclose no conflicts of interest.
This research was supported by grants from NIH/NIDCD: K08DC009631, P30-DC004661, and R01-DC005987-05.

METHODS

Animals

CBA/CaJ male mice, 4 to 6 weeks old, were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and maintained in the University of Washington Animal Care Facility. All procedures described have been approved by the University of Washington Animal Care and Use Committee.

Dissection

Mice were sacrificed by cervical dislocation. Each temporal bone is harvested manually with removal of the bulla and then placed into cold sterile culture medium (DMEM, 1% fetal bovine serum, 0.001% ofloxacin). Attached muscle fibers are removed, but neural tissue within the internal acoustic meatus is left intact (Fig. 1A). A small window in the thin bone overlying the utricle (easily identified through the transparent bone due to the white otoconia) on the cranial side of the temporal bone is carefully removed with a fine bone pick. Care is taken to leave the petrous ridge intact. Breaking the petrous ridge can destabilize the bony structure and lead to separation of the cochlea from the vestibular organs. Adequate bone over the vestibule is removed to expose the sensory epithelial surface of the utricle (Fig. 1B). The pigmented membranous labyrinth overlying the sensory epithelium (easily visible in pigmented mice) is then carefully removed with a number 11 scalpel blade and fine No. 55 Dumont forceps to expose the utricle to the culture media without disturbing the overlying otoconia. At no point is the utricle's epithelial or neural side actually touched by any dissecting instruments. Once the pigmented epithelium is opened, the dissection is complete, and the temporal bone is placed into a 48-well culture plate with 1 ml of culture medium at room temperature.

Cultures were maintained in a 48-well culture plate for 0 to 3 days in a 37°C incubator, maintained at 5% CO_2, changing culture media daily while nutating and rotating at 30 revolutions per minute, continuously. No neurotrophic factors were added to the culture media. Leaving the utricle within the temporal bone allows easy and atraumatic manipulation of the tissue as well as easy changing of culture media without fear of accidental aspiration of the utricle. Before fixation, otoconia are removed; a 25-gauge needle on a 3 ml syringe is used to gently blow off otoconia with a steady stream of culture media, whereas the utricle remains attached within the temporal bone. Again, leaving the utricle within the temporal bone during otoconia removal allows for much easier manipulation of the utricle without directly contacting the sensory epithelium. Tissue fixation is then performed by placing the temporal bone overnight in 4% paraformaldehyde at 4°C on a nutator. The fixed temporal bone is then rinsed in phosphate-buffered saline (PBS).

Only after fixation is the utricle actually handled with instruments. At this point, the utricle can be dissected free with much less risk of unintentionally damaging the sensory epithelium. Postfixation utricles are harvested from the temporal bone by carefully severing all connections to the membranous labyrinth and vestibular nerve and then grasping the utricle carefully on the neural side and removing it with fine No. 55 Dumont forceps.

Use of Ofloxacin in Media

Initial attempts at whole temporal bone cultures were unsuccessful because of high rates of infection. Culture and sensitivity analysis by the University of Washington microbiology laboratory identified several bacteria, including *Staphylococcus aureus*, *Enterobacter cloacae*, *Acinetobacter baumanni*, and *Proteus mirabilis*, all of which were sensitive to members of the fluoroquinolone family of antibiotics. To combat these bacteria, the fluoroquinolone ofloxacin (Sigma, St. Louis, MO, USA) was prepared as a 0.3% stock solution in PBS and added to culture media at 4 μl/ml for a final concentration of .001% ofloxacin. We found that ofloxacin is only required during the first 24 hours of culture to prevent infection for the duration of the culture period. We also tested whether 0.001% ofloxacin affected hair cell survival in free-floating utricle cultures and found no difference in survival.

Neomycin Damage Protocol

In the current study, utricles from in-bone preparations were cultured in 0, 1, 2, and 4 mM neomycin (Sigma) for 24 hours and then either fixed overnight or allowed to recover for

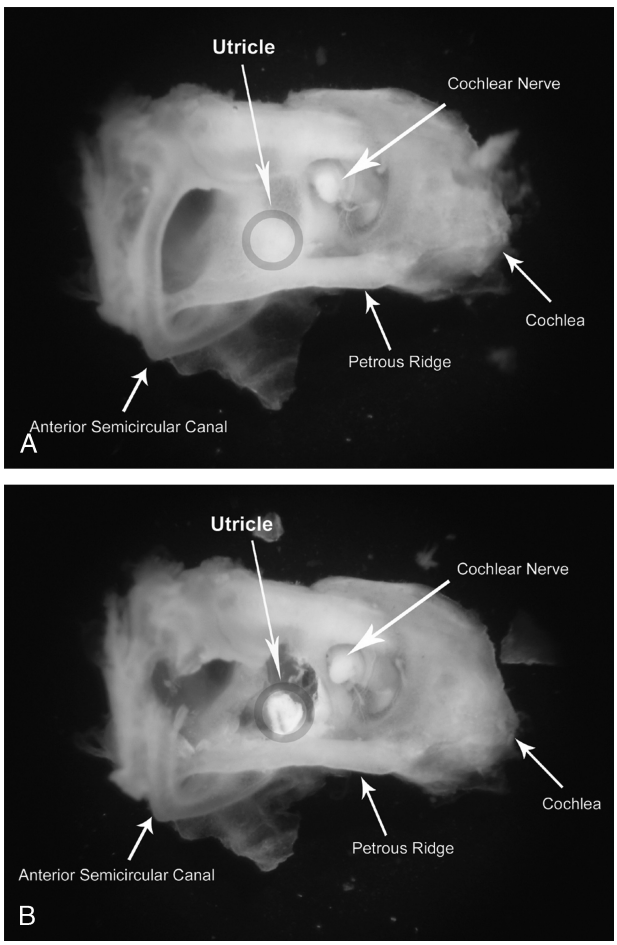

FIG. 1. Photomicrograph of mouse temporal bone before (*A*) and after (*B*) opening of window in bone overlying utricle. In *A*, the *circle* indicates location for bone removal overlying utricle directly adjacent to the petrous ridge. In *B*, the bone overlying the utricle has been removed with a fine bone pick or No. 11 scalpel blade, and the pigmented epithelium overlying the otoconia has been removed with fine forceps without touching the utricle or otoconia. The otoconia are undisturbed and visible overlying the utricular sensory epithelium. The petrous ridge is left intact as it helps maintain bony stability. The whole temporal bone is then placed directly into the culture well. The utricle sensory epithelium is not manipulated with any instruments until after the culture period and after fixation.

48 additional hours in DMEM/1% FBS before fixation in 4% paraformaldehyde for immunohistochemical staining.

Tacrine Protection Protocol

Previous studies have shown that tacrine exposure has a protective effect against neomycin exposure in free-floating utricles (8). We sought to determine whether similar results would be obtained using the in-bone method. In-bone utricles were exposed to 1 μM tacrine (Sigma) for 4 hours, followed by treatment with 0, 2, or 4 mM neomycin for 24 hours, with tacrine still present. After 24 hours of treatment, culture media was replaced with fresh media (DMEM, 1% fetal bovine serum) without neomycin or tacrine. Utricles were left in culture for an additional 48 hours after removal of tacrine and neomycin. Five utricles were used per experimental condition.

Hair Cell Counts

Otoconia were removed and utricles were fixed overnight in 4% paraformaldehyde, as described previously. After rinsing in PBS, the utricles were placed in blocking solution (2% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, and 0.4% Triton X-100 in PBS) for 3 hours at room temperature. Utricles were then double labeled using antibodies against calmodulin (monoclonal mouse, 1:200; Sigma) and calbindin (polyclonal-rabbit, 1:250; Chemicon, Billerica, MA, USA) overnight at 4°C (5). After additional PBS rinses, the utricles were incubated with secondary antibodies (Alexa 594 goat antirabbit IgG 1:500 and Alexa 488 horse antimouse IgG 1:500; Invitrogen, Carlsbad, CA, USA) for 2 hours at room temperature. Utricles were then washed in PBS and mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

Utricles were examined using either a Zeiss Axioplan II fluorescence microscope or an Olympus FV-1000 confocal microscope (Fig. 2). Hair cell counts from 4 randomly selected 900 μm^2 areas from the striolar and the extrastriolar regions were determined (Cunningham et al., 2002). For each utricle, hair cells in the 4 areas were totaled for striolar and extrastriolar regions separately. These hair cell totals were then averaged for the mean hair cell density in striolar and extrastriolar regions and are presented as a percentage of control utricles (± standard deviation [SD]) cultured without neomycin.

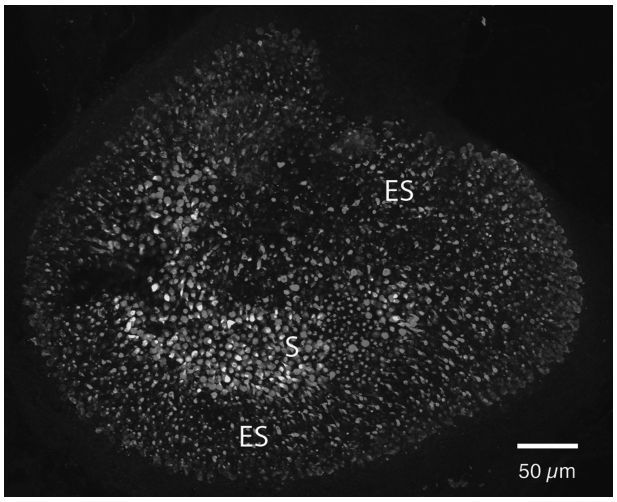

FIG. 2. Confocal image of mouse utricle cultured for 3 days and then fixed and labeled with antibodies against calmodulin (green) and calbindin (red) to delineate striolar (S) and extrastriolar (ES) hair cells. Hair cells are well preserved. Scale bar = 50 μm.

Free-Floating Utricle Cultures

For purposes of comparison to the in-bone method, utricles were cultured using the free-floating utricle method, as previously described (2,5). Mature mice (4–6 wk of age) were sacrificed by cervical dislocation. Utricles were then removed from the temporal bones using sterile technique and cultured in tissue culture plates with a 2:1 mixture of basal medium Eagle and Earle's balanced salt solution, with 5% fetal bovine serum (Invitrogen). Utricles were cultured for 24 hours with 0, 1, 2, or 4 mM neomycin. Five utricles were cultured per experimental condition. After neomycin treatment, otoconia were removed by a steady stream of phosphate-buffered solution (PBS), and then, utricles were fixed overnight in 4% paraformaldehyde at 4°C. After this, tissue was processed exactly as above. Utricles were also cultured for 24 hours with 0, 1, 2, or 4 mM neomycin in the presence of 0.001% ofloxacin to determine whether there was any effect of ofloxacin on hair cell survival in free-floating cultures.

Scanning Electron Microscopy of In-bone Utricles

Utricles prepared with the in-bone method were cultured for 1 hour or 72 hours in DMEM/1% FBS and then rinsed in PBS before fixation in 4% glutaraldehyde in 0.1 M sodium phosphate at 4°C overnight. Specimens were then rinsed 3 times in 0.1 M sodium phosphate buffer (PB) and then postfixed in 1% osmium tetroxide in 0.1 M PB for 30 minutes in an ice bath. Specimens were then rinsed in 0.1 M PB and dehydrated through a graded ethanol series: 35%, 70%, 95%, and 100% (×2). Utricles were then critical point dried, mounted on SEM stubs, and sputter coated with Au/Pd. Note that utricles were kept in situ within the temporal bone through processing and imaging. SEM was performed using a JEOL JSM-840A scanning electron microscope.

Statistics

All values were calculated and presented as the mean value ± 1 SD. Statistical analyses were performed using 1-way ANOVA (Vassarstats, http://faculty.vassar.edu/lowry/VassarStats.html) and 3-way ANOVA (PASW Statistics 18.0; SPSS, Inc., Chicago, IL, USA). Results were considered statistically significant if $p < 0.05$.

RESULTS

Utricular Hair Cell Survival in Culture Media

Adult utricles were cultured for 1 to 3 days in culture media. After 3 days in culture media, hair cell numbers were well preserved (Fig. 2). Hair cells were then counted from striolar and extrastriolar regions. Mean hair cell density per 1,000 μm^2 (SD) in striolar and extrastriolar areas was 11.0 (4.4) and 11.7 (1.9), respectively, after 24 hours of culture. After 3 days in culture media, mean hair cell density per 1,000 μm^2 in striolar and extrastriolar areas was 11.5 (3.1) and 14.7 (2.5), respectively, demonstrating favorable stability of hair cell density while in this culture condition (n = 5–9 utricles per group). Scanning electron microscopy of in-bone utricles fixed after 72 hours in culture demonstrated excellent stereocilia morphology when compared with utricles fixed after 1 hour in culture

(Fig. 3). Many hair cells exhibited blebbing of the apical plasma membrane after 1 hour in culture media. This seemed reduced after 72 hours in culture media, presumably because of acclimatization of hair cells to the media.

Neomycin Damage Protocol

Utricles were cultured for 24 hours with 0, 1, 2, and 4 mM neomycin using the in-bone method and then fixed for fluorescence microscopy. The mean (±1 SD) striolar and extrastriolar hair cell numbers expressed as a percentage of undamaged (no neomycin) controls are shown in Figure 4A. Hair cell counts after 24 hours in culture media with neomycin showed no significant hair cell loss in striolar or extrastriolar regions (striolar, $p > 0.20$ value; extrastriolar, $p > 0.20$, 1-way ANOVA). This is in contrast to marked dose dependent hair cell loss demonstrated with free floating utricles exposed to 24 hours of 1, 2, and 4 mM neomycin (Fig. 4A; striolar, $p < 0.0001$; extrastriolar, $p < 0.0001$; 1-way ANOVA). In addition, to determine whether ofloxacin was having a protective effect on hair cells, we also cultured free-floating utricles exposed to 0, 1, 2, and 4 mM neomycin with and without 0.001% ofloxacin for 24 hours and found no difference in hair cell survival ($p > 0.05$, 2-way ANOVA, data not shown).

Because no hair cell death was seen with the in-bone method at 24 hours, we examined whether hair cell death would emerge over a longer period. After rinsing away neomycin, utricles were cultured for 48 additional hours (24 h with neomycin followed by 48 hours in culture media without neomycin). Immunohistochemistry was performed to measure hair cell counts in striolar and extrastriolar regions. With this 72-hour protocol, there was significant dose-dependent hair cell loss. Figure 5B shows an example of a utricle cultured by this protocol after 4 mM neomycin compared with a utricle cultured for the same period but without neomycin exposure (Fig. 5A). Both striolar and extrastriolar hair cells in the in-bone cultures were dramatically affected (Fig. 4B; $p < 0.001$ for both striolar and extrastriolar hair cells, 1-way ANOVA). Interestingly, although at 24 hours, the 2 methods yielded dissimilar results, by 72 hours the in-bone damage protocol data closely matches with damage seen with free floating mouse utricles cultured for 24 hours with neomycin.

Tacrine-Induced Hair Cell Protection

We have previously identified tacrine as a drug that protects zebrafish lateral line hair cells and hair cells from cultured explanted utricles of mature mice against neomycin-induced hair cell death (8). To determine whether protective drugs found effective in our zebrafish screen and with free floating utricles were also protective with the in-bone method, hair cell counts were performed on utricles exposed to 2 and 4 mM neomycin with and without

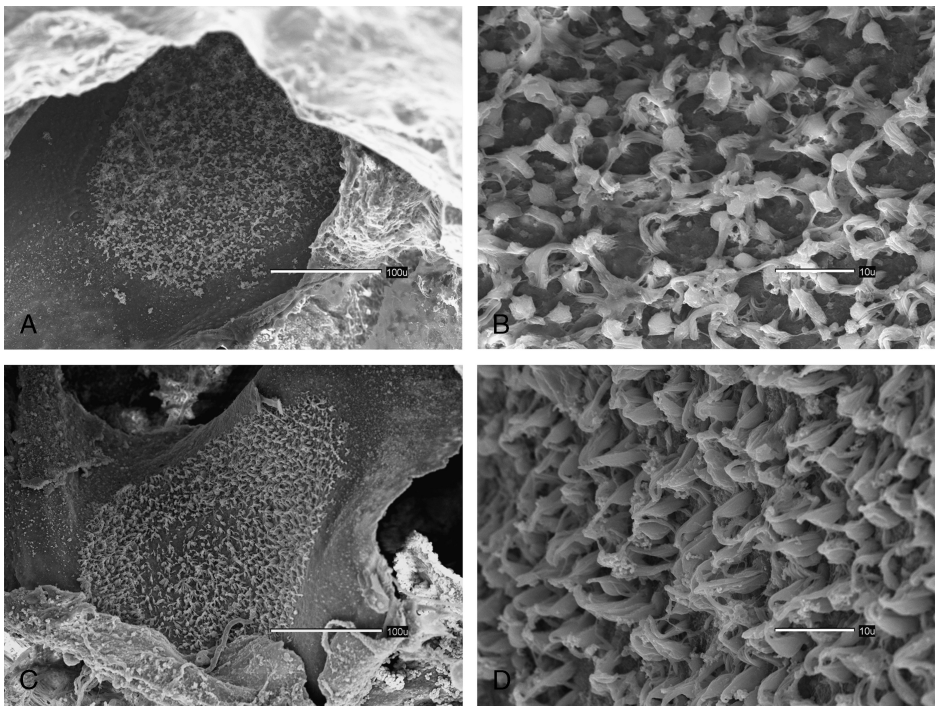

FIG. 3. Scanning electron micrographs of mouse utricles cultured using in-bone method. *A* and *B* demonstrate in-bone utricle after 1 hour in culture media. In *A*, the utricle sensory epithelium is easily visualized within the bony vestibule. At higher magnification (*B*), the stereocilia bundles are visible. Small blebs of the apical plasma membrane are seen and are likely because of the acute exposure to culture media. *C* and *D* demonstrate in-bone utricle after 72 hrs in culture media. After 72 hours in culture (*C*), the utricle sensory epithelium remains well visualized in the bony vestibule with excellent morphology of stereocilia seen at higher magnification (*D*). Blebbing has also decreased, potentially because of acclimatization of hair cells to culture conditions after 72 hours.

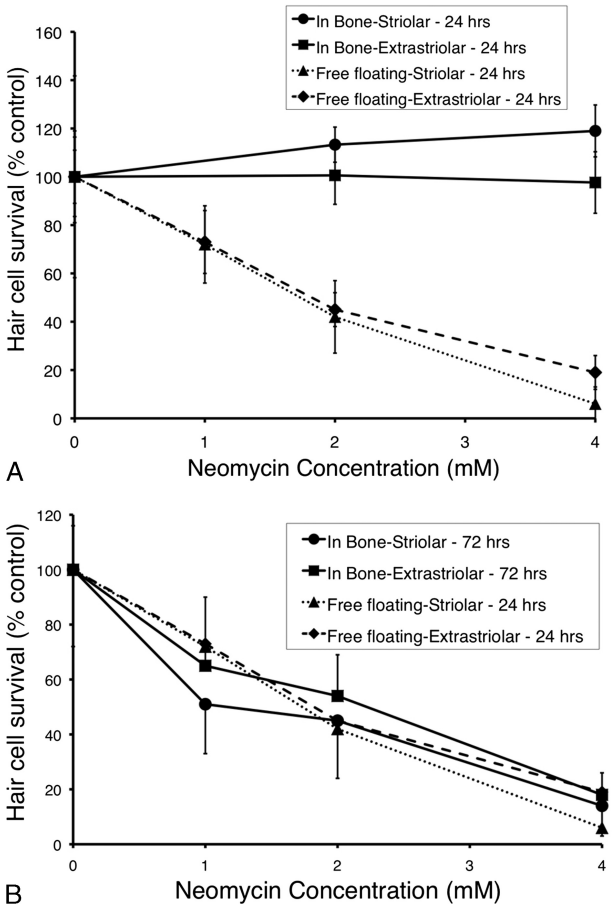

FIG. 4. Neomycin dose-response curves showing hair cell survival in utricles cultured for 24 hours (*A*) and 72 hours (*B*). *A*, Striolar and extrastriolar hair cell survival after 24 hours in vitro is compared between free-floating and in-bone utricle cultures. Utricles were exposed to 24 hours of neomycin at 0-, 2-, and 4-mM concentrations and then fixed and analyzed. *B*, In-bone cultures were exposed to neomycin for 24 hours and then rinsed and then cultured in fresh media (without neomycin) for an additional 48 hours. Hair cell loss for in-bone cultures maintained for a total of 72 hours are compared with data from free floating cultures, repeated from A. In *A*, in-bone striolar (*solid line* •) and in-bone extrastriolar (*solid line* ■;) there was no hair cell loss in response to neomycin. This is in comparison to the dose-response curves for striolar (*dotted line* ♦) and extrastriolar (*dashed line* ▲) hair cells in free floating utricles exposed to identical neomycin treatments. In *B*, after 24 hours of neomycin exposure, and 48 additional hours in culture without neomycin, in-bone utricles demonstrate increasing striolar (*solid line* •) and extrastriolar (*solid line* ■;) hair cell loss with increasing neomycin concentrations, more closely resembling the free floating utricle cultures after just 24 hours (*dotted line* ♦ and *dashed line* ▲). Data points represent the mean of 5 utricles for each experimental condition. Error bars represent the SD from the mean.

tacrine (Fig. 6). Tacrine pretreatment increased hair cell survival after 4 mM from 17% (mean hair cell density, 1.06 per 1,000 μm^2; no tacrine) to 46% (mean hair cell density 4.31 per 1,000 μm^2; with tacrine) in striolar regions of the utricle. In extrastriolar regions, tacrine pretreatment increased hair cell survival after 4 mM neomycin from 35% (mean hair cell density, 4.44 per 1,000 μm^2; no tacrine) to 57% (mean hair cell density 7.19 per 1,000 μm^2; with tacrine). This protective effect of tacrine was significant against both 2 and 4 mM neomycin in both striolar and extrastriolar regions ($p < 0.05$, repeated measures 3-way ANOVA).

DISCUSSION

Viable culture preparations from adult mammalian inner ear have been problematic. There is currently no satisfactory way to culture mature organ of Corti for a prolonged duration. As a result, most in vitro studies using adult inner ear tissue have avoided using cochlea, instead using free floating utricle explants, which survive nicely in culture (1,2,4,9). This technique, although effective, is technically difficult because of the size and fragility of the sensory epithelium.

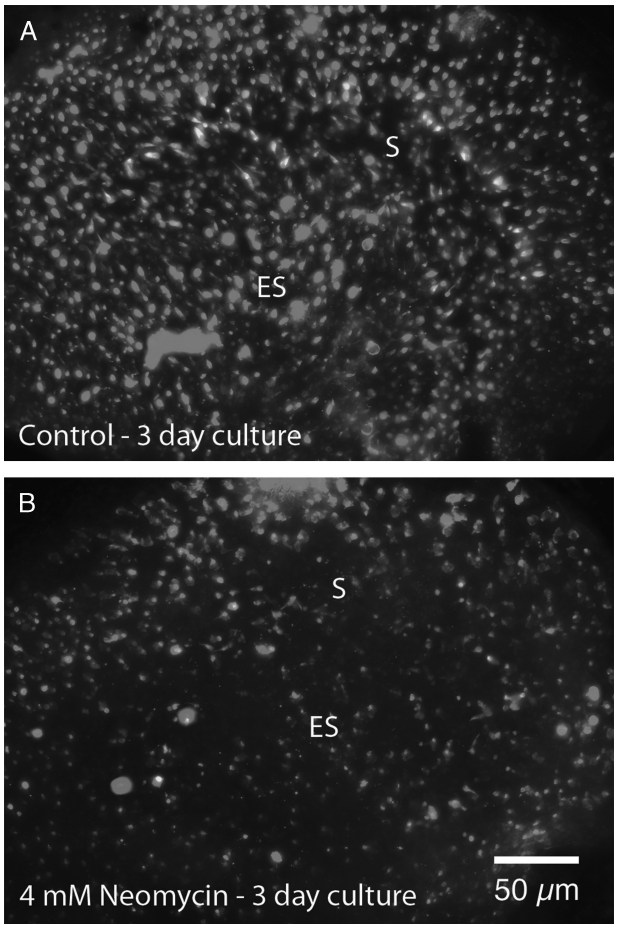

FIG. 5. Immunocytochemistry of neomycin-induced hair cell death in the mouse utricle cultured using the in-bone technique. Utricles were cultured for 24 hours with 0 (*A*) and 4 mM (*B*) neomycin, and then rinsed several times to remove neomycin and allowed to recover for 48 additional hours. Utricles were then fixed and labeled with antibodies against calmodulin (green) and calbindin (red). In *A*, there is excellent survival of striolar (red) and extrastriolar hair cells. In *B*, with 4-mM neomycin, there is marked striolar and extrastriolar hair cell loss. Scale bar in *B* = 50 μm.

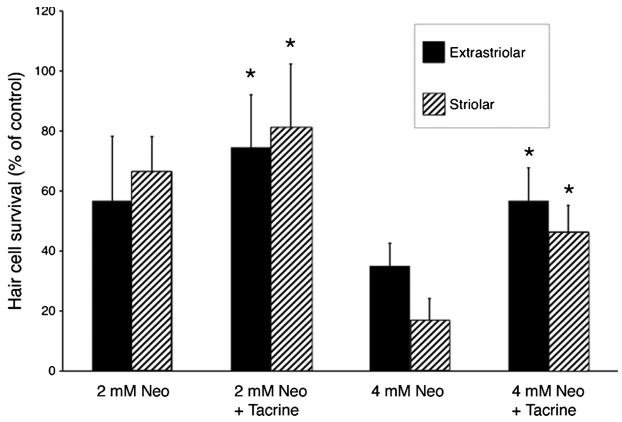

FIG. 6. Pretreatment with tacrine protects against neomycin-induced hair cell death in in-bone utricle cultures. Utricles were cultured using the in-bone method and then pretreated either with 0.01% DMSO control or 1 mM tacrine in culture media. Utricles were then treated with 2 or 4 mM neomycin for 24 hours followed by 48 hours in culture media alone without neomycin or tacrine. Pretreatment with tacrine significantly protected striolar and extrastriolar hair cells against 2 and 4 mM neomycin when compared with DMSO pretreatment controls (*$p < 0.05$, repeated measures, 6-way ANOVA). Data bars represent the mean of 5 utricles (black bars = extrastriolar hair cells, hatched bars = striolar hair cells). Error bars represent the SD from the mean.

The in-bone culture method we discuss here allows the adult mouse utricle to be harvested and left in culture for 3 days. Because the utricle is left within the temporal bone and not manipulated until after fixation, there is much less tissue damage and, thus, much more consistent tissue quality. In addition, removal of otoconia is significantly easier with the utricle left attached within the temporal bone. This alone makes this technique much more accessible and more easily taught to students and technical staff. In our experience, achieving expertise in the traditional utricle explant technique often can require months of practice, whereas the in-bone method can be mastered within days.

Aminoglycoside Damage Occurs Predictably

As with the free floating utricle cultures, aminoglycoside damage with the in-bone method occurs with a predictable dose-response relationship. However, it is interesting to note that damage occurs at a slower rate. With the in-bone method and neomycin exposure up to 4 mM for 24 hours, hair cell loss was not seen immediately but was very evident 2 days later, even when no neomycin was present during the final 48 hours of incubation. On the other hand, significant hair cell loss occurs after 1 day with free floating utricles in our study as well as others (5,6). Whether the in-bone culture method or the free floating method more closely resembles in vivo exposure conditions remains unclear. It should be noted that the time course of utricular hair cell loss after aminoglycoside exposure in vivo is not well described. Although most in vivo aminoglycoside damage protocols seem to require several days to cause damage (10), more recent studies demonstrate that aminoglycosides can be detected in the inner ear within hours after systemic administration (11,12). In addition, others have found that hair cell death occurs within hours after direct exposure of hair cells to aminoglycosides in vivo in guinea pig (12). It should be noted that in the in-bone culture, aminoglycosides would only have access to the apical side of the utricular hair cells, whereas free floating cultures, although still protected by otoconia, are circumferentially bathed in solutions containing aminoglycoside, potentially increasing the toxicity. Although unproven, one could also hypothesize that the in-bone culture method, by leaving neural connections relatively intact and sensory epithelium undisturbed, might result in hair cells that are less fragile than those in free floating utricles and thus less susceptible to aminoglycoside damage. These differences may account for some of the differences seen between the in-bone and free floating utricle cultures.

Difficulties With In-bone Method

Initial attempts at culturing whole temporal bones were hindered by difficulties with culture media infection. Microbiologic testing of the infected media yielded multiple organisms, all of which were sensitive to fluoroquinolone antibiotics. Cultures thereafter were thus cultured with 0.001% ofloxacin with excellent results and no apparent ototoxicity based on hair cell counts. It is notable that according to current recommendations from the American Academy of Otolaryngology–Head and Neck Surgery (13,14), only fluoroquinolone antibiotics are considered safe to use in the middle ear and ''less'' ototoxic. Nevertheless, to minimize any possible effect that ofloxacin might have on hair cell survival, culture media was changed after 24 hours to remove ofloxacin from the preparation. This initial 24-hour exposure to 0.001% ofloxacin was sufficient to prevent any subsequent infection during 5-day culture periods. In addition, to rule out the possibility that ofloxacin was protecting hair cells against neomycin, we exposed free floating utricles to neomycin with and without ofloxacin present and found no significant effect.

Caveats

The methods described here are meant to serve as an alternative to the free-floating preparation of the adult mouse utricle. Although there are advantages in terms of technical ease, difficulties with infection do require the addition of an antibiotic (ofloxacin) to the culture media. In addition, leaving the utricle within the temporal bone may expose cultured hair cells to additional survival factors generated by other tissues (bone, muscle, and nerves) in the culture media. This ''co-culture'' scenario could introduce more variability into the system, although in this study, the results from damaging and protective compounds were quite consistent. Clearly, the free-floating utricle preparation, as well as isolated epithelium preparations (15), remains valuable tools for investigating many questions about hair cell viability and regeneration.

Acknowledgments: The authors thank Dale Cunningham for excellent technical expertise in electron microscopy.

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