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# Pharmacologic Intervention for Acquired Hearing Loss: Assays of Drug-Induced Inner Ear Damage

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## Pharmacologic Intervention for Acquired Hearing Loss: Assays of Drug-Induced Inner Ear Damage

Hearing can be permanently impaired by therapeutic drugs, most notably the aminoglycoside class of antibiotics and the platinum-based chemotherapeutic drugs. The prevalence of this hearing loss in patients (ototoxicity) has prompted great interest in better understanding the mechanism of injury and prevention. Aminoglycoside ototoxicity usually affects hearing in the high-frequency range before progression to lower frequencies. For in-vitro studies, the organotypic organ of Corti model provides an inner ear (cochlear) tissue preparation for investigating the effects of drugs on the mammalian ear, including testing of drug toxicity, protective effects, and delineation of cellular and molecular mechanisms. Gentamicin is a prototype aminoglycoside, and a gentamicin injury model is presented here as an established preparation for testing potential protective drugs. Similar injury models can be developed using other ototoxic agents such as the chemotherapeutic drug cisplatin. For in-vivo investigation, the guinea pig is a preferred model. Two advantages of the guinea pig in hearing research include ease of access to the cochlea and a pattern of aminoglycoside-induced injury similar to humans.

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## Organotypic Organ of Corti Explant Model

### Purpose and Rationale

- This aminoglycoside-treated preparation allows to perform an in-vitro assay of drugs that may prevent injury to cochlear hair cells and thus serve as potential protective agents.
- A classic preparation involves exposure of the explant to gentamicin, creating a preferential outer hair cell loss, with a gradient of injury from the cochlear base to apex. This pattern of injury mirrors that observed in the human ear.
- An important feature of the model is its efficiency, in that it allows relatively rapid evaluation prior to investing the time and expense of in-vivo studies.
- The organotypic culture is derived from mammalian tissue and hence is physiologically relevant to the human cochlea.

### Procedure

#### Animal Selection

- CBA/J mice postnatal day 2–3 pups
  - Other strains and species, including transgenic mice, may be used depending on the experimental questions.
  - Guinea pig models are precocious in development and more closely resemble adult cochlea, making it challenging to maintain in culture compared to mice neonatal models.

#### Procedure

- Prepare culture dish
  - Prepare collagen gel: combine 180  $\mu$ L collagen I rat tail (BD Biosciences), 20  $\mu$ L of 10 $\times$  Basal Medium Eagle (Sigma), and 20  $\mu$ L of 2 % sodium carbonate.
    - Store on ice for 15 min after preparation.
  - For each cochlear explant, place one 15  $\mu$ L drop of collagen gel on 35-mm culture dishes.

- Allow to gel at room temperature for 10–15 min.
- Prepare 200 mL of serum-free medium as below and add 1 mL of solution to each culture dish.
  - 2 g bovine serum albumin (Sigma)
  - 2 mL Insulin-Transferrin-Selenium 100x supplement, (Invitrogen)
  - 4.8 mL of 20 % glucose (5 mg/mL) (Sigma)
  - 0.5 mL of penicillin G (8.8 U/mL) (Sigma)
  - 190.8 mL 1 $\times$  Basal Medium Eagle (Sigma)
  - 0.44 g sodium bicarbonate (2.2 g/L)
- Dissection
  - Mice pups are anesthetized and euthanized.
  - Inner ears and cochlea are extracted and placed in cold Hank's Balanced Salt Solution (HBSS).
  - Dissect away auditory nerve bundle and lateral wall tissues, including removal of stria vascularis while avoiding injury to the basilar membrane.
    - Avoiding trauma to the cochlear hair cells (located at periphery of the basilar membrane) will ensure quality of the preparation.
  - Meticulous care is required when placing the organ of Corti on previously prepared culture dish to ensure proper orientation.
  - The tectorial membrane should face upward. Take care to prevent it from being folded along the undersurface of the specimen, as this will obscure visualization. The explant should not be twisted or rotated but rather be allowed to have a gentle turn.
  - Incubate explants for 4 h at 37 °C, 5 % CO<sub>2</sub>, then add additional 1 mL of culture medium.
  - Continue incubation for 2 days to allow recovery from dissection stress.
    - Treat with drug of choice for 3 days.
- Videos describing the technique are available at:
  - <http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/video-tutorial-for-cochlear-dissection/>

- <http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/cochlear-dissection-summary/>
- Treatment
  - Incubate in the presence of the vehicle used in experimental group.
    - Care should be taken to control for the effect of solvents or vehicles.
      - DMSO, which is often used to dissolve putative protectants, has intrinsic antioxidant properties.
      - Similarly, phenol red frequently used in media also has antioxidant effect.
  - Replace medium with new media containing a concentration of gentamicin that creates a known gradient of injury.
    - *Note:* Commercial gentamicin is a mixture of three related compounds. Each batch therefore must be titrated to induce 50 % outer hair cell loss. The optimal concentration of gentamicin varies with lot but often falls in the 2–5  $\mu$ M range.
- Fixation and staining
  - Rinse explants 3 $\times$  with phosphate-buffered saline (PBS).
  - Fix with 4 % paraformaldehyde overnight at 4 °C.
  - Permeabilize for 30 min with 3 % Triton X-100 in PBS at 22–24 °C.
  - Rinse explants 3 $\times$  with PBS for 10 min.
  - Incubate with fluorescent phalloidin stain or Myosin VIIA antibody stain for 30 min.
  - Rinse explants 3 $\times$  with PBS for 10 min.
  - Mount on slide.

## Evaluation

- **Morphology**
  - Hair cells
    - Hair cells are examined under light or confocal microscopy to examine for hair cell death and structural changes from the apex to the base.
    - The hair cells are evaluated for integrity of the hair cell outline, stereocilia, and ultrastructural evaluation of organelles (mitochondria, nucleus, endoplasmic reticulum).
- **Ribbon synapse**
  - The presynaptic terminal can be identified with immunolabeling of CTBP2 (Kujawa and Liberman 2009).
  - Postsynaptic marker can be identified with immunolabeling using either PSD95 (postsynaptic density 95) or GluR2/3 (glutamate receptor subunits 2/3) (Yuan and Chi 2014).
- **Spiral ganglia**
  - Examination of the spiral ganglia cells requires different dissection and sectioning techniques, described by Szabo et al. 2014.
- **Cytocochleogram**
  - The number of preserved outer hair cells is counted from base to apex and entered into programs designed for cytocochleograms (KHRI Cytocochleogram is one such program available).
  - This provides comparison of cell counts to normative data established from control specimens.
  - Number of preserved hair cells is calculated as a percent and plotted as function of distance from apex to basal turn of explant.
- **Immunohistochemistry**
  - The explant has distinctive geometry, which must be preserved. The tissues are not thin sections but rather a complete tissue. Microscopy should consider vertical stacking and need to evaluate in two planes for nucleus and organelles.
  - Triton X-100 is the most commonly used permeability agent.
  - Immunohistochemistry is useful to examine specific molecules that may be up- or down-regulated.
- **Other approaches**
  - The sensory hair cells represent a small percentage of the cellular tissue within the explant, with most of the tissue mass contributed by supporting cells and neural elements. Therefore, sensitivity is limited for discerning effects on hair cells.

- **Western blotting** reflects this cellular mix. One protocol involves the following: use of glass/micro tissue grinder to pool and homogenize explants in lysis buffer. Follow general procedures for western blotting.
  - **Gene expression:** RT-PCR, microarray, and in-situ hybridization have been described for evaluation of gene expression. A variety of other techniques such as electroporation have also been described.
  - <http://www.jove.com/video/1685/primary-culture-plasmid-electroporation-murine-organ>
  - This video describes isolation and culture of the murine organ of Corti with or without the spiral limbus and spiral ganglion neurons. It also shows expression of an exogenous reporter gene in the organ of Corti explant by electroporation.
- metabolites. The impact of P450 activation/inactivation of drugs is not represented in the model.
- The endolymphatic potential is not preserved due to loss of boundaries between endolymph and perilymph.
  - Removal of stria vascularis and bypass of the blood labyrinth barrier increase sensitivity. The assay is thus highly sensitive to toxicities that might arise in the setting of a compromised blood–brain barrier, which occurs in a variety of disease states and with exposure to certain pharmacological stresses, such as chemotherapy.
  - Cochlear dissections may cause stress to the sensory hair cells (via temperatures, dissection method, etc.) and hence may cause expression of proteins that may lead to data modification (Casado and Cutillas 2011).

### Critical Assessment of the Method

- The organotypic organ of Corti model has a proven track record for predicting toxicity and potential protection for in-vivo experimentation.
  - Allows study of hair cell death and protection in a living cellular model
  - Highly sensitive assay for injury to outer hair cells or organ of Corti
- Assay is a starting point for screening and requires follow-up in-vivo experimentation for the assessment of likely clinical implications.
  - Some protective agents may overcome aminoglycoside toxicity by preventing drug entry or inactivating the parent compound; assessment for such effects using antimicrobial interference assays is a necessary step.
- Considerations
  - By virtue of removal of explant from intact organism, this model eliminates the first-pass effect and may not reflect normal metabolism/elimination kinetics of systemic applications.
    - The assay does not allow one to predict the toxicity of prodrugs or toxic

### Modifications of the Method

- Dissection model variations
  - A variety of animal alternatives to the mouse are available, most commonly including the rat or Mongolian gerbil.
  - Preparations can be made with or without the spiral limbus and spiral ganglion neurons.
- Immortomouse cell lines
  - Cell lines derived from the organ of Corti (immortomouse) have previously been explored as potential screening tools to evaluate toxic or protective effects on the inner ear.
  - These immortalized cells have been criticized for showing results that do not correlate with those observed in explant hair cells and in vivo.
  - These cells lack mechano-electrical transduction channels, possess molecular machinery that resists cell death from aminoglycosides, and typically require 100-fold increased dosing versus chronic explant model to elicit effects.

- Zebra fish models
  - These have been studied as screening tools for ototoxic agents and otoprotectants based on the premise that the mechanosensory cells found in neuromasts have similarities to sensory hair cells of the inner ear.
  - These neuromasts exhibit sensitivity to aminoglycoside and cisplatin ototoxicity that is analogous to that observed in the inner ear, although their use in screening has led to false positives and negatives. Results from zebra fish screens thus require verification in other systems.
- Pharmacologic variations: replacing the drug of interest
  - Other aminoglycosides
    - Streptomycin, kanamycin, tobramycin, neomycin, amikacin
  - Cisplatin
    - Platinum-containing chemotherapy agent commonly used for solid malignancies
    - Common toxicities include oto- and nephrotoxicity
- Chronic versus acute model
  - Chronic model example: 2.5–5  $\mu$ M aminoglycoside exposure for 72 h.
  - In contrast to the chronic treatment model, a 24 h acute treatment regimen typically involves gentamicin doses 10–100-fold higher.
    - Acute model may be useful for monitoring short-term changes in oxidative stress, protein expression, or covalent modifications.
- Toxicology screen: In assessing the inherent toxicity profile of a candidate otoprotectant, it is often helpful to establish the toxicity curve and use this data as a reference for defining target dosing for the putative drug.

### Examination of Electrode Insertion Trauma (EIT)

- Cochlear implant surgeries that involve surgical placement of an electrode array into the inner ear cause hair cell trauma, leading to reactive oxygen species formation, inflammation, apoptosis, and fibrosis in the traumatized area (Bas et al. 2012).

- These reactive measures diminish the effectiveness of the cochlear implant.
- The molecular mechanisms underlying hair cell apoptosis and inner ear fibrosis in a cochlear injury setting are not well understood.

### Procedure

- **Animals:** Postnatal day 3 rats (Wistar strain)
  - Animal is anesthetized and cochlea is dissected out.
- **Groups**
  - Control group: Cochleostomy only
  - Cochleostomy with 0.28 mm monofilament line insertion (EIT).
  - Cochleostomy with 0.28 mm monofilament line insertion and dexamethasone (DXM 20  $\mu$ g/mL).
  - Dexamethasone has been shown to be protective.
- **Cochleostomy**
  - *Procedure video link*
  - A 0.35-mm diameter hole is created in the cochlea with sharpened No.5 Dumont forceps next to round window.
  - Insert monofilament through hole three times at angles 110–150° to achieve appropriate insertion trauma into scala tympani.
  - Incubate cochlea for 10 min in phosphate-saline buffer.
  - Excise outer hair cell explants.
  - Culture in serum-free media (Dulbecco's Modified Eagle's Medium).
  - Supplement with glucose (6 g/L), 1 % of N-1 supplement.
  - Image using Wild-Heerbrugg M400 stereomicroscope and color camera.

### Evaluation of Electrode Insertion Trauma

- Reactive oxygen species detection (cellROX)
- Gene expression studies
  - Pro-inflammatory enzymes (iNOS, COX-2)
  - Pro-inflammatory cytokines (TNF03B1, IL-1 $\beta$ )
  - Wound-healing growth factors
- **Histology and morphology**
  - Morphology (see [Evaluation](#) section for more details)

- Cytocochleogram (see [Evaluation](#) section for more details)
- Immunohistochemistry: *Cleaved caspase-3*, *apoptosis-induced factor (AIF)*, and *endonuclease G (ENDO G)* (see [Evaluation](#) section for more details)
- Although animal models mirror human disease imperfectly, the intact organism preserves many of the physiologic features that are absent with in-vitro screens.
- The guinea pig model allows easy access to the cochlea and a pattern of aminoglycoside-induced injury that parallels the toxicity seen in humans.

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## Aminoglycoside In-Vivo Model

### Purpose and Rationale

- In-vivo aminoglycoside models allow investigators to examine the effect of aminoglycoside mechanisms on the inner ear and test possible therapies.

### Procedure

#### Animal Selection: Guinea Pig Model

- Pigmented adult guinea pigs weighing 200–400 g.
  - Albino guinea pigs carry mutations that affect auditory processing.
- Control: saline administration
- Test group: aminoglycoside administration
  - Gentamicin, aminoglycoside ototoxicity model, in the guinea pig in-vivo model is well established.
  - Generally aminoglycosides are well tolerated but body weight should be monitored.

### Procedure

#### • Treatment

- Aminoglycoside group
  - Inject 100 mg gentamicin base/kg body weight intramuscularly (subcutaneous and intraperitoneal are also available options).
  - Monitor body weight daily and adjust dose accordingly.
  - *Note:* Commercial gentamicin is a mixture of three related compounds. Each batch therefore must be titrated to induce 50 % outer hair cell loss.
- Treat daily for 14 days.
- Control group
  - Inject equivalent volume of saline in control group guinea pigs.
  - Treat daily for 14 days.
- Animals are monitored and evaluated at 12 weeks after beginning of treatment.

- **Testing**
  - **Auditory brainstem response (ABR) testing**
    - ABR is measured at scheduled intervals after ototoxic effects have stabilized.
    - ABR is used to determine auditory thresholds and also can yield latency and amplitude functions for more detailed analysis.
    - Animals are anesthetized by intramuscular injection of 40 mg/kg ketamine +10 mg/kg xylazine or equivalent drug combinations.
    - Needle electrode placement
      - The active electrode is placed at ipsilateral vertex.
      - The reference electrode is placed subcutaneous below ipsilateral right pinna.
      - Ground electrode placed either in opposite ear or right thigh.
    - Insert transducer speculum into either ear external auditory meatus, creating a closed acoustic system.
      - Drug-induced hearing loss is generally bilateral and thus it is only necessary to test one ear.
    - Present tone burst stimuli at low, mid, and high frequencies (for example, 4, 16, 32–48 kHz).
    - Output is fed to an amplifier, viewed on oscilloscope and stored for later evaluation.
    - Threshold shifts are calculated in comparison to individual prestudy established thresholds.
      - Threshold is defined as the intensity that produces a detectable change from the non-stimulus condition.
      - Thresholds are verified twice.
  - **Evaluation of vestibular function**
    - Place animals inside restraining box on rotation table.
    - Insert ground needle electrode to contralateral pinna and active electrode into periolcular region.
      - In the dark, induce nystagmus by abruptly starting or stopping rotational table.
      - Measure duration of post-rotary nystagmus and number of beats.
      - For a control, measure optokinetic nystagmus in the light.
  - **Endocochlear potential (EP)**
    - The EP is a positive voltage of 80–100 mV in the cochlear endolymphatic space.
    - EP is highest in the cochlear basal turn, decreased toward the apex.
    - It can be measured by passing double-barrel K-selective microelectrodes into the endolymph through the stria vascularis.
  - **Distortion product OAE (DPOAE)**
    - *Most commonly used method in rodents.*
    - DPOAE is evoked using a pair of primary tones,  $f_1$  and  $f_2$ .
    - Evoked frequency ( $f_{dp}$ ) is mathematically related to the primary frequencies.
    - Place spectrum analyzer microphone in the ear canal and record the amplitude of  $f$  and  $f_1$  and calculate  $2f_1-f_2$ .
  - **Dissection to examine cochlear morphology or immunocytochemistry**
    - Animals are anesthetized and sacrificed and cochlea dissected out.
    - For surface preparation and light microscopy
      - A small opening is made in the apical portion of the cochlea.
        - (Process analogous to the in-vitro explant prep; continue dissection and stain)
      - Fix with 4 % paraformaldehyde overnight at 4 °C.
        - Rinse 3× with phosphate-buffered saline (PBS) for 10 min.
        - Decalcify cochleae in 3 % EDTA at 4 °C for 2–3 days if necessary. In contrast to rodents, guinea pig cochleae can be dissected for surface preparations without decalcification.

- Rinse explants 3× with PBS for 10 min.
- 0.1 % Triton x-100 in PBS for no longer than 1 h.
- Rinse explants 3× with PBS for 10 min.
- Incubate with antibodies and/or stain.
- Rinse explants 3× with PBS for 10 min.
- Mount on slide.
- For electron microscopy
  - A small opening is made in the apical portion of the cochlea.
  - Perfuse cochlea through opening with
    - 0.1 M sodium cacodylate
    - 2 % glutaraldehyde
    - 2 mM calcium chloride
  - Incubate overnight at 4 °C.
  - Next, dehydrate cochleae with alcohol and embed in EMbed 812 resin.
  - Slice 6 mm para-modiolar planar sections and mount on slide with Richardson stain.
  - Examine under bright-field and differential interference contrast optics.

## Evaluation

- **Daily or weekly evaluation**
  - Body weight: Measure daily and adjust dose accordingly.
  - Evaluate blood urea nitrogen (BUN) and creatinine (Cr) for assessment of renal function, which is commonly impaired by aminoglycosides.
  - Serum albumin provides assessment of protein and nutrition.
- **Assaying of serum drug levels**
  - Obtain from control and treatment groups at desired times.
  - Only include animals who survived entire study period in analysis.
    - Retrieve blood via toenail clipping.
    - Centrifuge sample and store at −20 °C until analysis.
- For analysis, thaw and dilute 1:100 with normal saline.
- Analyze by conventional procedures.

## Histology and Morphology

### • Morphology

- Hair cells
  - Hair cells are examined under light or confocal microscopy to examine for hair cell death and structural changes from the apex to the base.
  - The hair cells are evaluated for integrity of the hair cell outline, stereocilia, and ultrastructural evaluation of organelles (mitochondria, nucleus, endoplasmic reticulum).
- Ribbon synapse
  - The presynaptic terminal can be identified with immunolabeling of CTBP2 (Kujawa and Liberman 2009).
  - Postsynaptic marker can be identified with immunolabeling using either PSD95 (postsynaptic density 95) or GluR2/3 (glutamate receptor subunits 2/3) (Yuan and Chi 2014).
- Spiral ganglia
  - Examination of the spiral ganglia cells requires different dissection and sectioning techniques, described by Szabo et al. (2014).
- **Cytocochleogram**
  - The number of preserved outer hair cells is counted from base to apex and entered into programs designed for cytocochleograms (KHRI Cytocochleogram is one such program).
  - This provides comparison of cell counts to normative data established from control specimens.
  - The number of preserved hair cells is calculated as a percent of total and plotted as function of distance from apex to basal turn of explant.
- **Immunohistochemistry**
  - The explant has distinctive geometry, which must be preserved. The tissues are not thin sections but rather a complete tissue. Microscopy should consider vertical



stacking and need to evaluate in two planes for nucleus and organelles.

- Triton X-100 is the most commonly used permeability agent.
- Immunohistochemistry is useful to examine specific molecules that may be up- or downregulated.

### Critical Assessment of the Method

- Other species successfully used in vivo include the chinchilla, gerbil, cat, as well as rodents.
  - Considerations in mice:
    - The drug of choice is kanamycin (Wu et al. 2001).
    - High dosage of aminoglycoside is necessary to achieve comparable ear injury state. However animal may develop renal insufficiency before achieving the desired gradient of hair cell injury.
  - Validity of the model is compelling, especially when protection observed from one in-vitro animal study produces similar results in a different in-vivo species model.
    - Effects of metabolism, absorption, excretion, and development of toxic metabolites (or inactivation) are likely to be more similar to the clinical setting than in the explant model.
- Allows for study of physiologic mechanisms for drugs that are promising in explant studies and protective mechanisms
  - In-vivo model allows examining the pathophysiology of clinical and extreme aminoglycoside dosages.
  - Aminoglycoside dosage necessary to induce extreme hearing loss (greater than 60 dB loss) may cause high morbidity rates.
  - Does conserve endocochlear potential (and fluid spaces of endo-/perilymph) which is lost in the explant model.
  - Effects on stria vascularis can be evaluated.
  - Effects on hair cell ribbon synapse and spiral ganglia can be studied.

### Modifications of the Method

- In-vivo models can vary in the species used, the aminoglycoside used, or the drug dosage examined.
  - Common drug variation is the chemotherapeutic agent, cisplatin
- Experimental models to eliminate all hair cells include:
  - Diuretic (ethacrynic acid or furosemide) + aminoglycoside
  - This model is not clinically relevant as it does not mimic a normal disease state.
- Procedure may be modified to examine aminoglycosides against a potential mitigator or aggravator.
  - Example of potential mitigators: antioxidants, modulators of cell death and survival pathways
- Combination models that examine dual insults (such as those that examine noise + aminoglycoside injury) may cause additive or synergistic injurious effects.

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