Video Article

A Comparative Study of Drug Delivery Methods Targeted to the Mouse Inner Ear: Bullostomy Versus Transtympanic Injection

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Abstract

We present two minimally invasive microsurgical techniques in rodents for specific drug delivery into the middle ear so that it may reach the inner ear. The first procedure consists of perforation of the tympanic bulla, termed bullostomy; the second one is a transtympanic injection. Both emulate human clinical intratympanic procedures.

Chitosan-glycerophosphate (CGP) and Ringer’s Lactate buffer (RL) were used as biocompatible vehicles for local drug delivery. CGP is a nontoxic biodegradable polymer widely used in pharmaceutical applications. It is a viscous liquid at RT but it congeals to a semi solid phase at body temperature. RL is an isotonic solution used for intravenous administrations in humans. A small volume of this vehicle is precisely placed on the Round Window (RW) niche by means of a bullostomy. A transtympanic injection fills the middle ear and allows less control but broader access to the inner ear.

The safety profiles of both techniques were studied and compared by using functional and morphological tests. Hearing was evaluated by registering the Auditory Brainstem Response (ABR) before and several times after microsurgery. The cytoarchitecture and preservation level of cochlear structures were studied by conventional histological techniques in paraformaldehyde-fixed and decalcified cochlear samples. In parallel, unfixed cochlear samples were taken and immediately frozen to analyze gene expression profiles of inflammatory markers by quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR).

Both procedures are suitable as drug delivery methods into the mouse middle ear, although transtympanic injection proved to be less invasive compared to bullostomy.

Video Link

The video component of this article can be found at https://www.jove.com/video/54951/

Introduction

Hearing impairment is the most frequent human sensory deficit and affects 5.3% of the worldwide population, and 30% of individuals over the age of 65 (http://www.who.int/topics/deafness/en, updated 2016). Hearing loss affects language acquisition in children and accelerates cognitive decline in older people. Therefore, it is a significant health-care problem with a tremendous socio-economic impact. It can be caused by genetic defects, environmental factors or a combination of both\(^1\), which in the end induce damage and death of hair cells and neurons in the cochlea. These cells do not regenerate in mammals, therefore cellular loss and concomitant hearing loss cannot be reversed. Clinical options are based on prosthetic devices, including hearing aids and cochlear, middle ear and bone conduction implants\(^2\). Unfortunately, there are no specific medical restorative treatments for hearing impairment and thus several research lines are focused on the development of preventive and reparative therapies. Novel therapeutic options include gene and cell therapies as well as development of small molecules for pharmacological therapy\(^2\).

One of the most important challenges in cochlear pharmacological therapy is drug delivery. Systemic treatments have limited efficacy in the cochlea due to the blood-labyrinth barrier\(^3\), continuous endothelium in contact with cochlear blood vessels, which acts as a physical and biochemical barrier to maintain inner ear fluid homeostasis, therefore limiting drug passage to the inner ear. It is permeable only to small
liposoluble molecules, although permeability can be increased during cochlear inflammation, and also with the use of diuretics or osmotic agents. The volume of drug that eventually reaches the cochlea after systemic administration is reduced; therefore, high doses that could cause organic toxicity are required. In addition, hepatic metabolism of the drug can produce toxic or inactive metabolites\(^4,5,6\). In contrast, local interventions allow the placement of a known limited quantity of the drug into the middle or inner ear without undesirable side effects\(^7,8,9\). In current clinical practice, intratympanic administrations are limited to certain cochlear pathologies, such as gentamicin in Meniere's disease\(^10\), corticosteroids in sudden deafness, Meniere's disease, immune-mediated and noise induced hearing loss,\(^11,12,13,14,15\) and insulin-like growth factor 1 (IGF1) in sudden deafness\(^1,16,17\). Formulations for local administration should preserve the delicate homeostasis (pH and osmolarity) of cochlear fluids. In addition, it is very important to maintain sterility throughout the process to avoid bacterial contamination of the cerebrospinal fluid. The excipient used for drug delivery should be biocompatible, nonototoxic and of the appropriate consistency. Liquid solutions are recommended for intracochlear injections, but are not suitable for the intratympanic route due to the clearance through the Eustachian tube. In this case, drugs are usually carried by semi-solid gels to increase their permanence in the middle ear\(^4,18,19\). Alternative delivery systems used as carriers to increase the passage of the drug to the inner ear are nanoparticles\(^20\) and adenoviruses\(^21\). Here we compared two vehicles: CGP and an RL solution. CGP is a hydrogel formed by chitosan, a linear polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine obtained from crustacean shells, and β-glycerophosphate, a polyol that forms a shield of water around the chitosan chains and maintains it in liquid form. CGP is thermosensitive and can be degraded by lysozymes, allowing a sustained drug release in the middle ear\(^22,23,24,25\). Chitosan-base hydrogels are suitable vehicles for clinical applications such as drug delivery due to their lack of immunogenicity and lack of activation of local inflammatory reactions\(^23,24\). On the other hand, RL buffer is a non-pyrogenic isotonic solution (273 mOsm/L and pH 6.5) intended for intravenous administration in humans as a source of water and electrolytes, especially in blood loss, trauma or burn injuries because the byproducts of lactate metabolism in the liver counteract acidosis.

Here we describe and compare two surgical methods that have been refined for local drug delivery to the mouse inner ear. The safety profile of both techniques was evaluated by using functional, morphological and molecular tests. Hearing was evaluated using Auditory Brainstem Response (ABR)\(^8,26,27\) performed before and after microsurgery at different times. End-point procedures were used to dissect the cochlea and compare the anatomical, cellular and molecular impact of these two microsurgical procedures.

### Protocol

Ensure that the animal handling procedures are in accordance with international and national regulations. The protocol follows the European Community 2010/63/EU and Spanish RD 53/2013 guidelines, respectively.

#### 1. General Animal Handling

1. Feed mice *ad libitum* with a standard diet and drinking water. Control health and well-being by following Federation for Laboratory Animal Science Associations (FELASA) recommendations.

#### 2. Hearing Assessment

NOTE: Track functional impact of microsurgical procedures by testing hearing before and many times after surgery (in this work 2, 7, 14 and 28 d postmicrosurgery) with non-invasive procedures such as ABR\(^2\).

1. For ABR testing, anesthetize mice with short duration effect protocols *i.e.* intraperitoneal injection of ketamine (100 mg/kg body weight (BW)) and xylazine (10 mg/kg, BW). Alternatively, perform hearing tests under inhalant anaesthesia.

NOTE: Since ABR parameters can be influenced by the anaesthetic protocol\(^28\), use the same one throughout the experiment.

2. Check the depth of anaesthesia by testing the toe-pincher reflex.

NOTE: When the withdrawal reflex disappears, the animal has reached an appropriate depth of anaesthesia to perform auditory testing.

3. Protect the eyes from desiccation and secondary *keratoconjunctivitis sicca* by topical administration of tear supplements, such as hydroxypropyl methylcellulose-based gels.

4. Keep the mouse at physiological temperature (37.5-38 °C) during the entire procedure. To avoid electrical interference, use a warm water pump and heating pads. Monitor body temperature with rectal probes. Always take care not to overheat the animal.

NOTE: We recommend cleaning the heating pad with a surface disinfectant between mice. For anaesthesia induction and recovery, electric heating pads, incandescent or infrared lights can be used.

5. ABR procedure

NOTE: For ABR registration, use a computer workstation with an inboard sound card to create waveforms (Digital to analog, D-A output, conversion) and to digitize electric response waveforms (analog to digital, A-D input), an attenuator, an oscilloscope and a low-impedance amplifier. Modern auditory workstations (*i.e.* Tucker Davis Technologies) include all these components in a single compact system.

1. Place the anesthetized mouse in prone position on the heating pads within a sound-attenuating chamber to avoid ambient noise interference and reverberation (Figure 1).

2. Deliver acoustic stimuli into the external auditory canal. Use preset stimuli or new signals designed with the appropriate software. Connect the D-A workstation output to the selected speaker.

NOTE: Free-field or closed-field speakers inserted in the external auditory canal could be used. The former are preferred when working with mice because of the difficulty in probe insertion and sound calibration in closed systems. Free field speakers stimulate both ears and elicit a binaural response. To obtain predominantly monaural responses with free-field speakers, contralateral activity has to be eliminated by occlusion (*i.e.* with ear plugs) or by masking the noise.
1. Place free-field speaker at a fixed distance (usually 5-20 cm) facing the head or selected ear with the center of the speaker aligned with the external auditory canal. Ensure that no obstacles are between the speaker and the ear and that the pinna is completely opened.

3. Place stainless steel subdermal needle electrodes as follows: i) the active (positive) electrode in the scalp between the ears (over the vertex of the skull), ii) the reference (negative) electrode, in the parotid region below the pinna, and iii) the ground electrode in the back, tail or hind limb region (Figure 1).

4. For ABR recording, generate broadband clicks and pure tone frequencies and present at decreasing intensities from 90 to 10 dB relative to the sound pressure level (SPL) in 5-10 dB SPL steps27,29,30.

5. Amplify, record and average the evoked electrical response to each stimulus and intensity. Use an amplifier with low noise and a good signal-to-noise ratio, and connect it to the A-D input.

6. Perform statistical analysis of ABR data using the appropriate software. Depending on the experimental design, use standard paired T-test or analysis of variance (ANOVA) to compare main ABR parameters in the different groups26,30.

3. Vehicle Preparation

1. Prepare and use vehicle solutions under sterile conditions.

2. Using a scalpel, make a 2 cm longitudinal incision from the mandible to the clavicle.

3. Place stainless steel subdermal needle electrodes as follows: i) the active (positive) electrode in the scalp between the ears (over the vertex of the skull), ii) the reference (negative) electrode, in the parotid region below the pinna, and iii) the ground electrode in the back, tail or hind limb region (Figure 1).

4. Check the electrical impedance in the positive and negative electrodes. Ensure that the impedance is less than 3 kOhm (ideally 1 kOhm). If it is higher, reposition, clean with alcohol or replace the electrodes.

5. Maintaining sterile conditions during the whole surgical procedure (sterile gloves, drapes, surgical instruments, etc.), insert the CGP-hydrogel into the middle ear cavity with a 27G needle or 25G insulin syringe and needle. Remove the outer layer of the middle ear mucosa using a curette or forceps. Lubricate the needle with mineral oil to avoid clogging.

6. Visually determine the ABR threshold during the test.

7. Perform data analysis either manually or automatically.

3. Microsurgical Procedures

1. Induce general anaesthesia with ketamine based combinations of sedatives and analgesics (i.e. ketamine 100 mg/Kg, medetomidine 0.05 mg/Kg and phentanile 0.025 mg/Kg) by intraperitoneal injection, followed by inhalant agents (i.e. isoflurane).

2. After administering injectable agents, adjust the anesthetic facemask to the mouse snout and connect the O2 supply to isoflurane vapor. Maintain the inhalation anesthesia during the microsurgery and monitor the anesthetic plane with the toe-pinching reflex and breathing pattern. Begin surgical preparation when the reflex is totally abolished and the mouse presents regular breathing.

3. Maintain body temperature with heating pads during the entire procedure and protect the eyes from corneal keratitis with a hydroxypropyl methylcellulose-based gel.

4. Prepare a clean surgical area by using sterile drapes. Sterilize the microsurgical instruments with a glass-bead sterilizer prior to surgery. Maintain sterile conditions during the whole surgical procedure (sterile gloves, drapes, surgical instruments, etc.).

5. Microsurgery

1. Bullostomy

NOTE: Bullostomy is a unilateral procedure. Operate one ear of the mouse and use the contralateral ear as the control.

1. Place the mouse in a decubitus supine position. Prepare the surgical area at the ventral surface of the neck using clippers to remove the fur. Clean the skin with povidone iodine based antiseptic solution, and cover it with sterile drapes.

2. Using a scalpel, make a 2 cm longitudinal incision from the mandible to the clavicle.

3. Under magnification with a surgical microscope, identify the submandibular glands and separate both with forceps. Retract the submandibular glands and localize the origin of the digastic muscle and the facial nerve.
4. Make an incision in the origin of the digastric muscle with a scissors, and retract it ventrally, exposing the underlying infero-medial aspect of the tympanic bulla.

5. Make an opening in the bulla by drilling into it with a 27 G needle (Figure 2A). Localize the stapedial artery and the RW membrane caudal to it (Figure 2B). Clean the blood from the drilled area with an absorbable gelatin sponge.

6. Using a 34 G catheter and a glass micro syringe, slowly inject 3-5 µL of vehicle solution (CGP-hydrogel or RL) through the bullostomy directly onto the RW niche, filling it (Figure 2C). Seal the bullostomy with 1-2 drops of tissue adhesive.

7. Return the submandibular glands to their initial position and close the skin incisions with 5-0 silk surgical suture. Apply a chlorhexidine-based antiseptic around the incision to avoid wound infection. NOTE: Absorbable and non-absorbable sutures could be used. Non-absorbable sutures must be removed in 2 weeks. Silk is not recommended for skin closure since its use is associated with incision infection and local tissue reactions.

2. Bilateral transtympanic injection

1. Place the mouse in the lateral decubitus position and prepare an aseptic surgical area below the external auditory meatus as described in 4.3.1.1.

2. Make a 0.5 cm longitudinal incision in the vertical part of the outer ear canal close to the tragus and section the internal cutaneous fold of the pinna (optional).

3. Locate the tympanic membrane at the end of the outer ear canal using a surgical microscope (Figure 2E) and identify the upper pars flaccida and the inferior pars tensa, which is divided into anterior and posterior sections by the handle of the malleus (Figure 2F).

4. Make a small myringostomy in the caudal portion of the pars flaccida. Make an additional incision in the pars tensa of the tympanic membrane, to allow air evacuation during injection. Gently inject 10-15 µL of vehicle (CGP-hydrogel or RL) solution with a glass micro syringe connected to a 34 G catheter through the pars flaccida, close to the RW niche until the middle ear is clearly full.

5. Close the skin incisions with a 5-0 silk surgical suture and clean as described in 4.3.1.7.

6. Place the mouse on its other side and operate the contralateral ear (steps 4.3.2.1-to 4.3.2.5).

4. Keep the mouse on a heating pad until it has regained sufficient consciousness to maintain sternal recumbency. Do not return an animal that has undergone surgery to the other animals until fully recovered.

5. Monitor the body condition, activity and the presence of signs of pain or stress. Provide analgesics if needed (i.e. Buprenorphine 0.05 mg/kg, Carprofen 5-10 mg/kg). Review the surgical wound daily and remove skin closures 7-14 days postoperatively after verifying that the wound has healed.

5. Morphological Evaluation of Cochlear Cytoarchitecture

1. Euthanize the mouse at the end of the experiment (in this work 28 days postmicrosurgery), with an intraperitoneal pentobarbital overdose (100 mg/kg) to study the long term effects of the surgery.

2. Perform a transcardial perfusion with cold 0.1 M Phosphate-buffered Saline (PBS), pH 7.5, followed by 4% (wt/v) paraformaldehyde (PFA) in 0.1 M PBS, pH 7.5 as described. CAUTION: Paraformaldehyde is highly toxic; avoid contact with skin, eyes or mucous membranes. Avoid breathing the powder during measuring and preparation.

3. Use a stereomicroscope to dissect out the inner ear from the temporal bone as described without separating the vestibular and cochlear components of the inner ear.

4. Fix the isolated inner ear with 4% (wt/v) PFA in 0.1 M PBS, pH 7.5 at 4 °C for 12 h with gentle shaking. Wash 3x for 5 min with 0.1 M PBS, pH 7.5.

5. Decalcify the samples with 10% ethylenediaminetetraacetic acid (EDTA) prepared in 0.1 M PBS, pH 6.5 at 4 °C for 10 d with constant shaking, changing the EDTA solution every 3 d.

6. When cochleae acquire a soft consistency, remove EDTA and wash 3x for 5 min with 0.1 M PBS, pH 7.5, with shaking at RT.

7. Embed the samples in paraffin wax as described and make 7 µm thick cochlear sections parallel to the modiolus.

8. To evaluate cochlear cytoarchitecture, stain sections with haematoxylin and eosin (H&E) and use a light microscope connected to a digital camera to capture images with 4X and 20X lenses.

6. Cochlear Gene Expression

1. Clean the working surface and surgical instruments with RNAse decontamination solution.

2. Euthanize the mouse as described in 5.1 and quickly dissect out the inner ear from the temporal bone using a microscope. Immerse the inner ear in a glass dish containing Ribonucleic acid (RNA) protector and stabilizer reagent.

3. Remove the remaining petrous bone with jeweller forceps and gently separate the cochlea from the vestibule using Vanna's eye scissors.

4. Immediately transfer the cochlea into a 2 mL microcentrifuge tube with 80 µL RNA protector and stabilizer solution and freeze the tissue by placing the tube in dry ice. Preserve cochlear samples at -70 °C until use.

5. Isolate cochlear RNA as described and determine its quality and quantity spectrophotometrically.

6. Generate cochlear cDNA from equal amounts of total mouse RNA using a reverse transcription commercial kit.

7. Perform qRT-PCR to amplify complementary deoxyribonucleic acid (cDNA) in triplicate to measure gene transcripts. NOTE: In this work pro- and anti-inflammatory gene transcripts of Il1b, Il6, Tgfb1, Tnfa, Il10 and Dusp1 were measured.

8. Calculate relative expression ratios by normalizing target transcript cycle threshold (Ct) levels to the arithmetic mean of the reference gene level and the relative quantification by normalizing the problem group transcript levels to the arithmetic mean of the calibrator group.
Representative Results

Hearing was tested by ABR before and at several times after microsurgery to evaluate the impact on auditory function (Figure 1A). ABR registers were performed under anaesthesia to avoid animal movement and voltage artefacts and therefore improve its reproducibility. Intraperitoneally administered ketamine based combinations or inhalatory isoflurane were usually employed to anesthetize animals during the ABR tests. The ketamine/xyalazine combination provides a short-acting (2-3 min) induction and a stable, safe maintenance phase while performing ABR registers. It should be noted that isoflurane can affect ABR measurement sensitivity. For ABR registers, subdermal electrodes are placed in specific locations (Figure 1B) and the electrical impedance is measured. If the impedance is 3 kOhm or higher, electrode positioning has to be checked to avoid alterations in ABR wave amplitude.

Intratympanic delivery is performed in mice by two microsurgical procedures (Figure 2). Exposure of the bulla during bullostomy involves retraction of the submandibular glands and digastric muscle. This procedure is carried out with extreme care because the carotid artery and vagal nerve are very close (Figure 2A). Next, the bulla is drilled to localize the stapedial artery and the RW membrane (Figure 2B). To avoid cracking bone, a small 0.5 mm aperture is made with a 27 G needle before drilling. The 34 G catheter is directed through the bullostomy towards the RW membrane and a small volume of vehicle is delivered onto the window niche (Figure 2C). The transtympanic injection is performed through an incision in the pars flaccida of the tympanic membrane with a 27 G needle; a larger one can provoke a tear in the membrane. Before the injection, we recommend making an additional incision in the pars tensa to allow the outflow of air during injection of the vehicle (Figure 2F). It is critical to avoid damage of the stapedial artery, a branch of internal carotid artery, which would lead to life-threatening bleeding.

Mice with bullostomy or transtympanic surgeries preserved hearing throughout the experiment, similar to non-operated controls (Figure 3). ABR thresholds in response to clicks and tone bursts did not change significantly after microsurgery compared to baseline values. No significant differences were observed between bullostomy and transtympanic approaches. Morphological studies were carried out to confirm correct vehicle delivery into the middle ear and to assess the potential changes caused by the procedures in cochlear cytoarchitecture. None of the main cochlear regions showed morphological alterations and animals from both procedures presented a similar morphology of all cochlear structures (Figure 4A). In addition, the cochlear profiles for gene expression of pro- and anti-inflammatory cytokines were also studied. Despite lack of functional differences in ABR data between the two procedures, bullostomy caused a stronger inflammatory response than the transtympanic approach (Figure 4B).
Figure 1. Experimental Design and Hearing Assessment. (A) Diagram of the experimental procedure. Hearing was evaluated with ABR before and after microsurgery. Cochlear samples were obtained 28 days after microsurgery. (B) Anesthetized mouse in prone position over the heating pad inside a sound-attenuating chamber, with subdermal electrodes placed in the scalp between the ears over the vertex of the skull (active, positive); in the parotid region below the pinna (reference, negative) and in the back (ground). The free-field speaker is placed at a fixed distance (5 cm) facing the right ear. Please click here to view a larger version of this figure.
Figure 2. Microsurgery for Vehicle Application. (A) Ventral view of the tympanic bulla. The bullostomy is performed caudal to the facial nerve with a 27 G needle. (B) RWN and stapedial artery can be observed through the perforation. (C) The 34 G catheter is directed through the bullostomy toward the RW niche. (D) One month after the bullostomy, a small bony scar is present in the opening site (arrowhead). (E) Lateral view of the ear, showing the incision in the external ear canal and the tympanic membrane (square). (F) Detail of the tympanic membrane. A puncture was made at the caudal upper quadrant of the tympanic membrane using a 27 G needle (black asterisk, in the pars flaccida); the injection was made through this perforation using a 34 G catheter. An additional hole was made in the cranial inferior quadrant of the membrane (white asterisk, in the pars tensa) prior to the injection to balance the tympanic pressure. (G) View of the 34 G catheter through the puncture of the tympanic membrane. (H) View of the cochlear region 24 h after the microsurgery. RWN filled with vehicle solution (asterisk). Lat, lateral; Ro, rostral; Do, dorsal; Ma, malleus; Co, cochlea; OW, Oval window; RWN, round window niche. Scale bars = 200 µm in A, D, F; Scale bars = 100 µm in B, C, H; Scale bars = 1,000 µm in E, G. Please click here to view a larger version of this figure.
Figure 3. Hearing Assessment. Evolution of ABR thresholds (mean ±SEM, in dB SPL) in response to click (A) and tone burst (B) stimuli, before and 7, 14 and 28 days after micro-surgery in male eight-week-old C57BL/6J mice. Bullostomy (orange; n = 11); transtympanic injection (blue; n = 6); non-operated (grey; n = 11). Please click here to view a larger version of this figure.
Figure 4. Cochlear Morphology and Gene Expression Analysis. (A) Morphology of the main cochlear structures at the base of the cochlea. Haematoxilin-eosin staining of mid-modiolar paraffin sections (7 µm), of ears from non-operated mice, and mice one month after microsurgery intervention by bullostomy or transtympanic injection. The scala media compartment (a,b,c) presents all the main components. Details of each of these structures (numbered boxes) are shown in the subsequent images: spiral ganglion (1), organ of Corti (2), spiral ligament (3) and stria vascularis (4). Inner hair cell (asterisk); outer hair cells (head of arrow). Scale bars = 100 µm in a,b,c; Scale bars = 50 µm in a-1,2,3,4. (B) Cochlear expression of inflammatory markers 28 d after the microsurgery. Comparison between bullostomy (orange) and transtympanic injection (blue) and to non-operated mice (white). *: non-operated vs. operated groups; ^: comparison between operated groups. Gene expression levels are represented as $2^{-\Delta\Delta C_{\text{t}}}$, or the n-fold difference relative to non-operated group. Values are presented as mean ± SEM of triplicates from pool samples of 3 mice per condition. Statistical significance: **$p \leq 0.01$; ***$p \leq 0.001$; **$p \leq 0.01$; ^^^$p \leq 0.001$. Please click here to view a larger version of this figure.

Discussion

Local drug delivery to the inner ear can be done directly by intracochlear injection or indirectly by intratympanic administration, placing the drug in the middle ear. Intracochlear administration provides controlled and precise drug delivery to the cochlea, avoiding diffusion through window membranes, basal-to-apical concentration gradients and clearance through the Eustachian tube. However, it is usually a highly invasive procedure that requires a complex and delicate microsurgery. In this context, the industry is developing new, coated, implantable devices for sustained drug release. On the other hand, intratympanic administration is a minimally invasive and easy to perform procedure that allows the injection of larger volumes of the drug into the middle ear, although the pharmacokinetics is not easy to control. The majority of the
drug is cleared through Eustachian tube and the remaining fraction has to diffuse through the RW membrane to reach the cochlea\(^{18}\). RW is the site of maximum absorption of substances from the middle ear into the perilymph-filled tympanic duct of the cochlea\(^{2}\). It is a semipermeable three layer structure, although its permeability depends on the drug characteristics (size, concentration, solubility and electrical charge) and on transmembrane transport systems (diffusion, active transport or phagocytosis)\(^{42}\). The oval window and otic capsules are alternative but less effective entrances to cochlea\(^{3,44}\).

Here we demonstrate and compare two microsurgical methods for targeted drug delivery into the mouse middle ear: bullostomy and transtympanic injection procedures. Common critical steps to these procedures include: i) an evaluation of hearing before and after the microsurgery, ii) preparation of a homogeneous vehicle solution under sterile conditions, iii) careful supervision of the anaesthetic procedure and monitoring of animal body temperature and constants, iv) slow placement of the appropriate volume of vehicle targeting the RW, and iv) taking cochlear samples to complete molecular and morphological analysis.

Retroatricular and ventral approaches for bullostomy have been described\(^{7,43}\). We used the ventral approximation because in our experience it has resulted in less morbidity and provided better access to the RW\(^{46}\). Transtympanic injections are usually carried out through the pars tensa of the tympanic membrane, anterior or posterior to the malleus manubrium\(^{12}\). In this work we performed a modification of the technique, an injection through the pars flaccida beyond the malleus with a previous additional puncture of the pars tensa to allow air evacuation during injection.

The transtympanic injection was less invasive than the bullostomy, although both microsurgeries were rapid (20 and 5 min per ear for bullostomy and transtympanic approach respectively), with short postoperative recovery times and no morbidity. Most importantly, both procedures maintained hearing and the ABR parameters were identical to those determined before the microsurgery. The transtympanic approach takes less time than the bullostomy and can be performed in both ears of the same animal during the same intervention. Advantages of the transtympanic injection are thus that it can be performed bilaterally and repeated, if required. On the other hand, bullostomy provides direct visual access to the RW membrane and allows the filling of the RW niche. In contrast, transtympanic injection does not allow for control of vehicle placement in the RW niche.

The procedures reported in this work describe how to perform a local drug vehicle delivery to the middle ear for pre-clinical applications such as evaluation of ototoxicity and evaluation of efficacy in hearing loss. Two microsurgery procedures are described that provide alternative methods with specific advantages and drawbacks. Both preserve hearing and do not cause morphological alterations. Local inflammation is described as a potential complication of bullostomy. A set of complementary techniques are also described for postsurgical procedures, including hearing, morphological and inflammatory marker expression evaluations. Future applications for these techniques include the preclinical evaluation of new therapies for hearing loss, including genetic, cellular and pharmacological approaches, in animal models. Intratympanic administrations ensure the delivery of the treatment in the middle ear, in contact with the round window membrane, facilitating the passage into the perilymph without evident cochlear damage.

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