Time-related Aspects of Otoprotection

Experimental Studies in Rat

ADNAN LIDIAN
Abstract


Intratympanic injection of various otoprotectants through the round window membrane (RWM) might become available in the near future as an alternative to the currently available medical and surgical methods used to treat several inner ear diseases. The most common outcome of such diseases is sensorineural hearing loss (SNHL).

Two examples of these otoprotectants are Edaravone and Brain-Derived Neurotrophic Factor (BDNF), both of which have already proved effective against noise-induced hair cell loss, barotrauma and ototoxicity caused by cisplatin. In four different studies we used two electrophysiological methods, auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE), to study the effects of tobramycin and Pseudomonas aeruginosa exotoxin A (PaExoA) on the inner ears of 129 male Sprague-Dawley rats.

In two investigations, not only the otoprotective effects of Edaravone on tobramycin-induced ABR threshold shifts and PaExoA-induced DPOAE threshold changes, were studied but even different application times, in order to establish in which interval it was still possible to achieve effective otoprotection. We found that Edaravone gave otoprotection from tobramycin when injected simultaneously or within 7 days, but it had only a limited effect on the changes in DPOAE thresholds caused by PaExoA when injected 1, 2, or 4 hours after the exotoxin.

The effect of BDNF on PaExoA-induced ABR threshold shifts was investigated in two studies, where different doses of intratympanically injected PaExoA were used and where BDNF was applied simultaneously, 12 or 72 hours after exotoxin instillation. We found that BDNF had an otoprotective effect on SNHL induced by different doses PaExoA when injected simultaneously or with no more than 12 hours delay.

Keywords: sensorineural hearing loss, Edaravone, brain-derived neurotrophic factor, auditory brainstem response, Sprague-Dawley rat, distortion product otoacoustic emission, Pseudomonas aeruginosa exotoxin A.

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Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world.

Albert Einstein

To my family
List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:


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<td>AOM</td>
<td>acute otitis media</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>COM</td>
<td>chronic otitis media</td>
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<td>N-methyl-D-aspartate</td>
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<td>OM</td>
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<td>OME</td>
<td>otitis media with effusion</td>
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<td>PaExoA</td>
<td><em>Pseudomonas aeruginosa</em> exotoxin A</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RAOm</td>
<td>recurrent acute otitis media</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
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<tr>
<td>RWM</td>
<td>round window membrane</td>
</tr>
<tr>
<td>SNHL</td>
<td>sensorineural hearing loss</td>
</tr>
<tr>
<td>TrK</td>
<td>tyrosine kinase</td>
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</table>
Introduction

Otitis media (OM) is broadly defined as inflammation of the middle ear from any cause. Acute otitis media (AOM) constitutes the rapid onset of middle ear inflammation with its attendant acute signs and symptoms. AOM is extremely common, its highest incidence occurring in the 6-month age group with 75% of all infants suffering one episode by 12 months of age. Moreover, infants who suffer their first episode of AOM before 12 months are more susceptible to recurrent AOM (RAOM) than those who do not. RAOM means four or more episodes of AOM occurring during a 1-year period. In USA, OM is the most common reason for visiting a pediatrician and is the disease most commonly treated with antibiotics. Overall, health care costs in USA attributable to OM amount to approx. 4 billion $ annually [1].

If for some reason fluid accumulates behind an intact tympanic membrane, this condition is referred to as otitis media with effusion (OME). The etiology underlying OME is not yet fully understood, but evidence suggests Eustachian tube dysfunction, viral infection, or inflammatory sequelae of AOM as possible causes. Should the effusion persist for more than 3 months, OME is defined as chronic.

Although prevalence rates of OME approach 90% in some populations, studies have shown that the vast majority of cases of AOM and OME resolve spontaneously and without sequelae [2].

When complications do occur, their sequelae can cause significant morbidity and occasionally even mortality. In general, the sequelae of AOM can be divided into two categories. Extracranial complications comprise chronic suppurative otitis media (CSOM), conductive hearing loss, sensorineural hearing loss (SNHL), mastoiditis, chronic otitis media with perforation, facial paralysis and cholesteatoma. The second category, less common, includes intracranial complications such as meningitis, brain abscess, epidural abscess, lateral sinus thrombosis, otitic hydrocephalus and cavernous sinus thrombosis [3].

Hearing loss and otitis media

AOM, RAOM and CSOM can be associated with temporary or permanent threshold shifts, as detected by auditory brainstem response (ABR) and
reduced otoacoustic emission (OAE). Hearing loss is more common at high-frequency.

The exact mechanism of hearing loss in OM has not yet been elucidated; however, one proposed mechanism is that repeated episodes of bacterial infection with transmission of bacterial toxins will affect the basal turn of the cochlea via the round window. It is the hair cells at the basal turn that are responsible for high-frequency hearing.

Pathogenesis of AOM and CSOM

Risk factors for AOM include obstruction and dysfunction of the Eustachian tube (e.g. adenoid hypertrophy, sinusitis, nasopharyngeal carcinoma), immunodeficiency whether primary or acquired, congenital midfacial anomalies (e.g. cleft palate, Down syndrome), ciliary dysfunction and gastro-esophageal reflux [4].

Environmental factors, such as absence of breastfeeding in infancy, passive exposure to tobacco smoke, number of hours spent in child daycare and low socioeconomic status, have all been associated with a higher OM rate.

For CSOM the risk factors include a history of RAOM, parents with a history of COM, large families with more siblings and crowded daycare premises [5]. No study has yet established a link between CSOM and gender, breastfeeding or passive smoking.

Allergy has been considered a risk factor since some studies showed allergens to cause nasal and Eustachian tube obstruction. On the other hand, studies involving antihistamines, pointing to an allergic cause of OM, have failed to establish any beneficial effect in OME [6].

Heritability studies suggest that there is a substantial genetic element (40-70%) in the risk of RAOM or COM. To date, only a handful of the genes underlying this genetic susceptibility have been identified. These include several regions of linkage on chromosomes 3p25, 10q22, 10q26,17q12 and 19q13 identified by two genome-wide linkage scans, which appear to harbour susceptibility loci. Detailed mapping of these regions has yet to identify the causative genes [7].

Viral infection, especially respiratory syncytial virus (RSV), commonly found in AOM and OME isolates, is regarded as a co-pathogenic factor in OM from the standpoint of specific Eustachian tube dysfunction and generalized upper airway edema [8].
Microbiology in AOM and CSOM

Bacteria can reach the middle ear via the external auditory canal when there is a tympanic membrane defect or reflux through the nasopharynx. From the middle ear, bacteria and their toxins can reach the inner ear via the round window and in this way lead to inner ear impairment and hearing loss.

It is well established that *Streptococcus pneumoniae* is the most common microorganism isolated from AOM samples, followed by *Haemophilus influenzae*, *Moraxella catarrhalis*, and group A streptococci. These same microorganisms have been isolated from chronic OME samples, using polymerase chain reaction (PCR) technique, demonstrating at the very least the presence of bacterial genomic DNA in effusion samples [9].

In CSOM the bacterial isolates differ from those found in AOM or chronic OME. Aerobic and anaerobic bacteria are present in CSOM isolates. The most common aerobic bacteria isolated are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and other Gram-negative bacilli such as *Escherichia coli*, *Proteus* species, and *Klebsiella* species [10]. *P. aeruginosa* is known to reside in the moist environment of the external auditory canal, whereas *Staphylococcus aureus* is usually found within human nares. *Bacteroides* and *Fusobacterium* species are the most common anaerobic bacteria isolated in CSOM.

Pseudomonas aeruginosa and PaExoA

The genus Pseudomonas contains more than 140 species, most of which are saprophytic. Most Pseudomonas known to cause diseases in humans are associated with opportunistic infections. *P. aeruginosa* and *P. maltophilia* account for approx. 80% of isolates from clinical specimens. Because of the frequency with which it is involved in human disease, *P. aeruginosa* has attracted the most attention. *P. aeruginosa* is the most frequently isolated bacterium in CSOM reported from different parts of the world. It is an ubiquitous free-living, Gram- negative, aerobic, round opportunistic human pathogen having unipolar motility. Although seldom causing disease in healthy individuals, it is a major threat to hospitalized patients, especially those with cancer or burns. The high mortality associated with *P. aeruginosa* infections is attributable to a combination of weakened host defenses, bacterial resistance to antibiotics and the production of extracellular bacterial enzymes and toxins.

*Pseudomonas aeruginosa* Exotoxin A (PaExoA) is an exotoxin produced by most strains of *P. aeruginosa*. It is a highly ototoxic 70 kD protein that can damage the inner ear, either reversibly or irreversibly, as has been proved morphologically [11] and electrophysiologically [12].
PaExoA as a protoxin binds to the α-2 macroglobulin receptor and enters cells by endocytosis. The toxin then cleaves into a larger 45 kD receptor binding domain and a smaller 27 kD enzymatically active domain that inhibits protein synthesis by blocking elongation factor 2 [13]. PaExoA toxicity is comparable to α-toxin of Clostridium perfringens and to diphtheria toxin. The clinical effects of PaExoA, given i.v., are a decrease in cardiac output and systemic blood pressure, with death several hours later following a period of respiratory failure and metabolic acidosis. At sublethal doses it causes epithelial, endothelial, stoma cell and skin necrosis [14]. The primary target area in the inner ear is not yet known, but stria vascularis appears to be a very early target for Haemophilus influenzae endotoxin [15].

In a previous study, Takumida and Aniko showed that various routes can be exploited by bacterial exotoxins to gain access to the inner ear, e.g. the round window, blood vessels or lymphatics, and/or by interscala exchange, causing a disturbance not only of the cochlea but also of the vestibular end organs [16].

While sporadic reports have been published on Pseudomonas labyrinthitis [17] and on PaExoA-induced osteomyelitis of the temporal bone [18], the most common complication when PaExoA remains in the middle ear whether for a longer time, or briefly, is hearing loss. For this reason we used PaExoA as a model for CSOM in our studies.

Round Window Membrane

The round window membrane (RWM) is a triple-layered structure that protects the inner ear from middle ear pathology and facilitates active transport. The structure of the RWM consists of an outer epithelium of low cuboidal cells lining the middle ear, a central connective tissue layer consisting of fibroblasts, collagen and elastic fibers, and an inner epithelium of squamous cells interfacing with the scala tympani.

The human RWM is 60-70 µm thick but thickens following a middle ear infection. The human RWM differs from, and yet bears similarities to, that of other species. General similarities include the observation that it is basically a triple-layered, as has been reported in chinchilla, guinea pig, domestic cat and rhesus monkey. The average thickness of the feline RWM of domestic cat (20-40 µm) and of rhesus monkey (40-60 µm) is much more akin to that of the human than is the membrane thickness of rodents (10-14 µm in chinchillas).

The functions of the RWM include releasing mechanical energy and/or conducting sound to the scala tympani, participating in absorption by and secretion from the perilymph and is involved in the defense system of the inner ear [19].
RWM permeability

A large variety of molecules are able to cross the RWM, including various antimicrobials, corticosteroids, anaesthetics, ions and macromolecules, including bacterial toxins. RWM permeability is regarded as an accident rather than a function of the membrane conferred by its anatomical characteristics, location and frequent occurrence of middle ear disease.

Many factors contribute to the permeability of the RWM, including the size, molecular weight, charge, the morphology of the compound, and RWM thickness. Regarding the size of molecules, 1µm spheres can cross the RWM, but not 3 µm spheres. Substances with a molecular weight of less than 1000 kD diffuse swiftly across the RWM, whereas substances over 1000 kD are transported by endocytosis.

The charge of the substance can also influence its ability to traverse the RWM; for example, it has been noted that cationic ferritin can, but anionic ferritin cannot. An increase in RWM thickness will reduce its permeability [20].

Although all these factors that affect the permeability of the RWM have long been known, it is still not entirely clear if there is a definite correlation between its structure (morphology) and its degree of permeability, thus affecting inner ear function. This is of the utmost importance as the RWM is now regarded as an important therapeutic approach route for the treatment of the inner ear diseases. Anatomic variations of the round window (RW) niche have been found in approx. 30% of human temporal bones [21]. Silverstein et al. [22] reported from their surgical observation that only 71% of RW niches were entirely patent, 17% were partly obstructed and 12% were completely blocked. This may explain some of the problems associated with local substance delivery to the inner ear.

Ototoxicity

Ototoxicity can be defined as damage to the inner ear by a toxic substance. Damage can occur in the cochlea, vestibular apparatus, and auditory nerve. Some of the substances known to be associated with ototoxicity are shown in Table 1.
<table>
<thead>
<tr>
<th>Type/group</th>
<th>Name of oxotoxic substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycoside antibiotics</td>
<td>Gentamycin, streptomycin, tobramycin, neomycin, netilmicin, kanamycin, amikacin, dihydrostreptomycin, ribostamycin</td>
</tr>
<tr>
<td>Non-aminoglycoside antibiotics</td>
<td>Vancomycin, erythromycin</td>
</tr>
<tr>
<td>Loop diuretics</td>
<td>Furosemide, ethacrynic acid, bumetanide, torsemide</td>
</tr>
<tr>
<td>Chemotherapeutic agents</td>
<td>Cisplatin, carboplatin, nitrogen mustard</td>
</tr>
<tr>
<td>Salicylates</td>
<td>Aspirin</td>
</tr>
<tr>
<td>Anti-malarial drugs</td>
<td>Quinine, chloroquine</td>
</tr>
<tr>
<td>Chemicals and other substances</td>
<td>Lead, mercury, carbon monoxide, arsenic, carbon disulfide, tin, hexane, toluene, alcohol</td>
</tr>
</tbody>
</table>

**Table 1.** Common substances known to be associated with ototoxicity

The most commonly used ototoxic substances are drugs used to treat life-threatening diseases where other options have failed or are unavailable. Other ototoxic substances are bacterial toxins, such as, PaExoA.

Both auditory and vestibular symptoms can occur in ototoxicity, whether the ototoxic substance has been applied systemically or topically.

The symptoms of cochlear ototoxicity due to systemic administration include bilateral sensorineural hearing loss and tinnitus. These symptoms are similar to those seen in systemic toxicity but are limited to the treated ear. Hearing loss in one ear produces less severe consequences than bilateral, because usually only one ear is necessary for adequate functioning, in most hearing conditions.

Vestibular ototoxicity due to systemic administration of an ototoxic substance includes dizziness, loss of the sense of balance, faintness, unsteadiness, and/or nystagmus.

Normal vestibular functioning relies on symmetrical input from both ears; for this reason, symptoms of vestibular ototoxicity following topical administration may be more severe as the injury to the vestibular system is asymmetric.

All the above symptoms can be temporary, disappear after the toxic substance has been cleared from the body, or they can be permanent.

In the case when hearing loss due to a toxin or other ototoxic substance becomes permanent, the first detectable symptom is an increased auditory threshold for high-frequency sounds.
Morphological and electrophysiological changes

Morphologically although not invariably, the outer hair cells (OHC) of the organ of Corti are commonly the first cells to exhibit signs of ototoxicity. This early OHC loss can be found in both human tissue and animal models.

The damage to these cells ranges from damaged stereocilia on the surface of the hair cells to complete loss of hair cells leaving only supporting cells.

In more severe damage, morphological studies showed even loss of inner hair cells (IHC) with their associated spiral ganglion cells.

In electrophysiological studies the first effect of ototoxic substances can manifest itself as a reduction in evoked otoacoustic emissions (OAEs) and/or auditory brain stem responses (ABR). The reduction is first seen in high-frequency responses. Although in some other cases, such as after carboplatin administration, degeneration begins inside the IHC. Carboplatin affects the peripheral and central nervous system and can damage the auditory nerve. In this case, OAEs may be normal, but abnormalities will be seen in ABR. This type of deficit is termed auditory neuropathy.

Aminoglycoside ototoxicity

Aminoglycoside antibiotics are one family of drugs that have been recognized as potentially ototoxic. Earlier studies suggested an incidence of aminoglycoside-related hearing loss when used in therapeutic doses in up to 14% of treated subjects [23]. More recent studies indicate an incidence of 5-7% [24][25].

The most frequently used aminoglycosides are gentamycin, streptomycin, tobramycin, neomycin, kanamycin, netilmicin and amikacin. These drugs are used to treat severe infections caused by aerobic Gram-negative bacteria and P. aeruginosa. Aminoglycoside antibiotics are bactericidal and exert their therapeutic action by inhibiting bacterial protein synthesis. Aminoglycosides bind to the bacterial 30S ribosomal subunit and block initiation of protein synthesis, cause misreading of the mRNA, or facilitate premature termination of ongoing translation of mRNA template [26].

In plasma, the half-life of aminoglycosides is about 2-3 hours, and the drugs are eliminated by glomerular filtration. In the inner ear, aminoglycosides can be detected within a few minutes of systemic application, and can remain in the endolymph and perilymph for at least 30 days [24].

Hair cells are the cellular target for aminoglycoside antibiotics in the ear. OHCs seem to be the most vulnerable to attack, but IHCs too can be affected by prolonged drug use or by higher doses. The loss of hair cells caused by aminoglycosides occurs via apoptosis [27].
Two forms of apoptosis are currently known: extrinsic death receptor-mediated apoptosis and intrinsic mitochondrial-mediated cascade. The later, considered to be the main pathway, is induced by aminoglycosides in the cochlea. In this pathway, generation of reactive oxygen species (ROS) is believed to be the initiating step of aminoglycoside ototoxicity in a cascade of events that ultimately result in cell death. The formation of ROS by aminoglycosides appears to involve iron and can generate superoxide free radicals and lipid peroxides [28].

Otoprotection and otoprotectors

Over the past few years many in vitro and in vivo studies have provided a revealing insight into the mechanism whereby hair cells are protected from ototoxic attack.

Several studies have demonstrated that the use of substances such as iron chelators, antioxidants, and neurotrophic factors can protect or restore hair cells when a rescue attempt is made before or immediately following an ototoxic attack [29,30, 31].

Theoretically, otoprotectors can be divided into two groups of substances, depending on the way these agents exert their otoprotective effect. The first group comprises NMDA receptor blockers, calcium channel blockers, ROS scavengers, corticosteroids and NOS inhibitors. These probably work by blocking signal routes at different levels, causing oxidative stress and ultimately hair cell damage [32]. The second group includes neurotrophins and caspase or calpain inhibitors. They may be closely related to the apoptotic process [33].

Since the otoprotective effect of these agents is influenced by the timing of their administration, it is important to determine which is the optimal time for their application in order to achieve optimal protection or rescue.

Neurotrophins

Neurotrophic factors (NTFs) are peptides that are produced and then secreted by neurons, sensory cells, glial cells and muscle fibers. Neurotrophins are the most frequently expressed and therefore the most extensively studied of all NTFs.

The following types of neurotrophins have been identified in mammals: Brain-derived neurotrophic factor (BDNF), Nerve growth factor (NGF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5) also called Neurotrophin-4 or (NT-4). All of these neurotrophins bind to p75 neurotrophin receptor, but have a second ligand-specific receptor in the
tyrosine kinase (TrK) family: NGF binds to TrkA, BDNF and NT-4 bind to TrkB and NT-3 binds to TrkC [34].

The two most important neurotrophins in the inner ear are BDNF and NT-3.

These peptides, present in the developing rat inner ear, are known to prevent neuronal death [35] and are even essential for the maintenance of inner ear function [36]. In experimental studies, BDNF and NT-3 have been shown to protect the inner ear from both noise-induced hair cell loss and ototoxicity caused by cisplatin [37].

In our studies we investigated different time protocols after intratympanic application of an exogenous BDNF against PaExoA ototoxicity and its influence on ABR threshold shifts.

**Edaravone**

Edaravone (MCI-186, 3-methyl-1-phenyl-pyrazoline-5-one), a free radical scavenger, can interact with both peroxyl and hydroxyl radicals to form oxidized compounds [38]. It had a potent scavenging effect on free radicals in experimental studies on ischaemia [39]. In Japan, Edaravone is an approved neuroprotective drug for the attenuation of brain ischaemic damage [40]. It has been marketed solely in Japan by Mitsubishi Pharma since 2001. Furthermore, Edaravone has already been used for the clinical treatment of acute myocardial infarction [41] and rheumatoid arthritis [42]. In recent studies Edaravone was shown to have a therapeutic effect on inner ear barotrauma in the guinea pig [43] as well suppressing streptomycin-induced vestibulotoxicity [44]. In view of these findings, we carried out two studies, the first to establish if Edaravone could be used to prevent cochlear damage caused by tobramycin. The second was to ascertain if Edaravone could prevent the changes in DPOAE thresholds caused by PaExoA when it was applied intratympanically.
Aims of the present investigation

1. to assess the otoprotective efficacy of Edaravone against aminoglycoside ototoxicity.
2. to investigate whether a single dose of BDNF is an effective otoprotective against various doses of PaExoA when used simultaneously.
3. to determine whether BDNF has an otoprotective effect on ABR threshold shifts induced by 15 µg PaExoA when given 12-72 hours after the exotoxin.
4. to study the early effect of PaExoA on distortion product otoacoustic emission (DPOAE).
5. to assess the otoprotective effect of Edaravone against PaExoA ototoxicity.
Material and Methods

Animals

129 outbred, male, albino Sprague-Dawley rats with body weights of 200-250g were used in Papers I, II, III and IV. They were purchased from a local breeder (B &K Universal AB, Sollentuna, Sweden).

The animals were allowed to adapt to the conditions prevailing at our laboratory for at least 1 week before measuring commenced. The animals were housed in Plexiglas cages (45x22x20 cm) on woodchip bedding, in a temperature controlled room (20-22°C), humidity 40-50%, ambient noise level <40 dBA and a 12/12 hour lighting cycle. The rats were fed ad lib on Beekay Feeds standard diet. The Swedish National Board for Laboratory animals gave approval for the experimental series for laboratory animals (C276/98) & (131/08).

Anaesthetics

Prior to each intratympanic injection and/or ABR measurements as well as DPOAE measurements the animals were anaesthetized with a combination of ketamine (Ketalar, Parke-Davis) (90 mg/kg) and xylazine 10 mg/kg (Rompun vet., Bayer) as an initial dose administered i.p. If needed, a supplementary dose of ketamine (30 mg /kg) was given.

Tobramycin

Tobramycin (Nebcina, Meda) was injected subcutaneously (s.c) at a dose of 160 mg/kg b.w. (Paper I).

Edaravone

Edaravone powder (Mitsubishi Pharma) was donated by Professor Takumida, Hiroshima University, Japan. Edaravone solution was prepared by dissolving the dry powder in 1N NaOH, adding sterile water and 1N HCl to pH 7.0. A fresh preparation was made every day, as Edaravone is unstable.
in solution. Edaravone was injected intraperitoneally (i.p) at a dose of 3 mg/kg b.w or in the same dose intravenously (i.v.) (Paper I). In Paper IV Edaravone was given as intratympanic injection at a dose 3 mg/kg b.w.

**Pseudomonas aeruginosa Exotoxin A (PaExoA)**

PaExoA was obtained from Sigma-Aldrich Sweden AB. The middle ear was exposed to a single dose of PaExoA. Doses at various concentrations of PaExoA, diluted in 20 µl saline, were instilled into the right middle ear cavity through a minute puncture by Metal Tip® (0.4 x 60 mm) needle in the posterior-superior quadrant of the tympanic membrane (Papers II, III and IV).

**Brain-derived neurotrophic factor (BDNF)**

Brain-derived neurotrophic Factor (BDNF) (Sigma-Aldrich Sweden AB) applied intratympanically in doses of 4 µg/20 µl simultaneously with the PaExoA in treatment groups and as a single dose in the control group (Paper II). The same dose of BDNF was given 12 and 72 hours after PaExoA in the treatment groups (Paper III).

**ABR procedure (Papers I, II and III)**

ABR technique was used, consisting of 1024 frequency-specific tone bursts at nine frequencies (2, 4, 6, 8, 10, 12, 14, 16 and 20 kHz) with a trapezoid envelope of 5 ms overall duration. The plateau was 3 ms, with rise and fall times of 1 ms each. These were presented monaurally, at a rate of 16/s through a 3 mm bore plastic tube inserted into the right external auditory meatus. Potentials were recorded via three subdermally placed platinum needle electrodes inserted at vertex (reference), right mastoid (negative), and left hind limb (earth) using a Grass P5 amplifier.

A PC utilizing software from Tucker-Davis Technologies averaged the responses. The ABR threshold was defined as the lowest intensity of sound stimulus (in 5-dB steps) that induced a detectable, reproducible wave-II response. The frequency-specific ABR was measured before instillation (control value) and then after instillation (treatment values) according to different time protocols. A complete description of the techniques utilized for data recording is published elsewhere [45].
OAE procedure (Paper IV)

Distortion product otoacoustic emissions (DPOAEs) were recorded using the TDT BioSig II system. All stimuli were digitally synthesized using TDT SigGen software applications. The ratio of F2 to F1 was 1,225 and L1 was held 10 dB SPL above L2. Signals were then passed through TDT ED1 speaker drivers and then EC1 electrostatic loudspeakers. DPOAE measurements were recorded using an ER10B + low-noise microphone and probe housed in the same coupler as the F1 and F2 speakers. A fast-Fourier transformer (FFT) was performed by the TDT BioSig software on the resulting output in order to obtain the magnitude of the 2F1-F2 distortion product. Distortion product amplitude measurements were obtained for F1 levels ranging from 35 to 80 dB SPL in 5-dB SPL steps. DPOAE thresholds were defined as an emission that was 3 dB greater than the noise floor. The latter was typically between -5 and 0 dB SPL. DPOAE thresholds were measured at nine frequencies 6, 8, 10, 12, 16, 20, 24, 28 and 30 kHz. We divided those tested frequencies into three regions that correlate closely with frequency-specific regions of cochlea as follows: low-frequency region (6-10 kHz), medium frequency region (12-20 kHz) and high-frequency region (24-30 kHz).
Results

1. Effect of tobramycin/Edaravone on ABR threshold shifts (Paper I)

Part one

Animals given i.v. Edaravone once daily for 3 days did not show any ABR threshold shifts Group A.

In Group B, repeated s.c. injection of tobramycin and simultaneously saline i.p. once daily for 14 days produced ABR threshold shifts with some individual variability; one rat showed only high-frequency loss; 2 rats a slight flat loss and 4 a severe flat loss. Hearing threshold deterioration was 5.3 dB SPL mean loss on day 7 after the first tobramycin + saline injection. On day 14 it reached 15.4 dB SPL, and continued with 22.8 dB SPL loss on day 21 and 28.8 on day 35 (Paper I, Fig.1).

In Group C, rats given s.c. tobramycin and simultaneously i.p. Edaravone showed no change in ABR threshold shifts.

Statistically the means of the thresholds for groups B and C were compared; the first significant changes appeared on day 7 at 2, 4, and 6 kHz. A further significant difference was noted on day 21 at all frequencies except 8 kHz and in all but 2 and 4 kHz on day 28.

On day 35 (the last ABR measurement) all thresholds rose significantly in the tobramycin group, when compared with the tobramycin + Edaravone group.

Part two

The control Group D, given repeated s.c. injections of tobramycin and simultaneously i.p. saline once daily for 14 days, showed some hearing loss, although less pronounced than group B.

The rats given 10 s.c. injections of tobramycin and two i.p. of Edaravone delayed to 7th and 8th day respectively after tobramycin Group E, showed no changes in hearing thresholds.

When Edaravone injections were delayed to 14th and 15th day after tobramycin Group F, only a temporary but significant protection effect was
noted on days 21 and 28. On day 35, hearing loss was just as serious as in the non-protected group (Paper I, Fig. 2).

2. Effect of PaExoA and simultaneous BDNF injection on ABR threshold shifts (Paper II)

Control groups

Intratympanic injection of a single dose of saline or BDNF Groups A and B did not produce any statistically significant ABR threshold shifts on days 2, 5, and 15 after injection.

All rats in control groups C, D and E, given different intratympanic doses of PaExoA, showed hearing impairment. The ABR threshold shifts measured on days 2 and 5 after instillation of PaExoA alone varied between 3 and 27 dB SPL.

By day 15 there was slight recovery of hearing, especially at low frequencies, in all control groups. Thus the ABR threshold shift in all these rats appeared to be temporary.

Consequently, an extra ABR measurement was performed on day 21 in those rats given the highest dose of PaExoA (10 µg or Group E). ABR threshold shifts had recovered by day 21, especially at frequencies 2, 4, 6, 8, and 10 kHz, but still equaled 10 dB SPL or even more at frequencies 12, 14, 16, and 20 kHz (Paper II, Fig. 1).

Treatment groups

The rats in treatment groups 1, 2, and 3 exposed to 1, 2, and 10 µg PaExoA respectively, and at the same time given 4 µg BDNF, showed no significant ABR threshold shift when compared with controls (Paper II, Figs. 2, 3, and 4).

The Mann-Whitney U-test was used to compare the ABR threshold shifts in treatment groups versus control groups. The p-values from this test proved statistically significant for all frequencies on days 2, 5, and 15 (Paper II, Figs. 5, 6, and 7). The only exceptions were the p-values for 4 and 12 kHz on day 2 (Paper II, Tab III).
3. Effect of PaExoA + BDNF (after 12 and 72 hours) on ABR threshold shifts (Paper III)

Control groups

Higher doses of PaExoA were used in paper III. The rats in control groups 1, 2, and 3 received 15, 20, and 25 µg PaExoA as a single intratympanic injection.

In Group 1 the mean ABR threshold shift had changed on day 7, when compared with control values ranging between 11 and 14 dB SPL. There was still a deterioration of SNHL on day 14, where the mean ABR threshold shifts varied from 12 up to 18 dB SPL. On day 21 the changes in mean ABR threshold shift had decreased by 7 up to 11 dB SPL, but by the end of the study (day 35) held steady in the range 7-10 dB SPL.

In Group 2, greater changes in mean ABR threshold shift were observed vis-à-vis control values. By day 7 the changes ranged between 12 and 22 dB SPL, while on day 14 they ranged from 21 up to 35 dB SPL. By the end of the study the mean ABR threshold shift changes ranged between 20 and 24 dB SPL.

In Group 3 we used our earlier time protocol where the ABR measurements were made at c (control), then 2, 5, 15, and 21 days after inoculation. In this group, the mean ABR threshold shift changes on day 2, when compared with control day, showed a range of 23-40 dB SPL, remaining high on days 5, 15 and 21 (Paper III, Fig.1).

When we compare the mean ABR threshold shifts between control Groups 1 and 2 at different times, we see that they were more significant in group 2 or when we gave a larger dose of PaExoA (Paper III, Fig.2).

Treatment groups

Rats given 15 µg PaExoA and then 4 µg BDNF 12 hours later (Group A), showed some changes in mean value when compared with controls. On day 7 the mean shift values ranged between 4 and 9 dB SPL. We observed that the changes were greater at 12, 14, 16 and 20 kHz frequencies, ranging between 8 and 9 dB SPL. At the lower frequencies, 2, 4, 6, 8 and 10 kHz, the mean ABR threshold shifts ranged between 4 and 5 dB SPL. There were still changes in mean shift values on day 14, ranging between 4 and 8 dB SPL. On day 21 and later, day 28, the shift values started to diminish; they ranged between 0 and 4 dB SPL on day 21 and between 0 and 3 on day 28.

By day 35, the mean ABR threshold shifts had returned to control values for all frequencies (except 12 kHz) where the change was only 2.5 dB SPL (Paper III, Fig.3A and B).

In Group B, rats received 15 µg PaExoA and then 4 µg BDNF 72 hours later, mean ABR threshold shifts changed on day 7, compared with control
values, ranging from 15 up to 19 dB SPL. On day 14 this range was between 13 and 19. Measurements on days 21 and 28 showed a range of 11 up to 20 dB SPL. On the final day of the study the range was 10 up to 19 dB SPL (Paper III, Fig. 4A and B).

4. Effect of PaExoA on DPOAE thresholds (Paper IV)

Single intratympanic injection of 15 or 20 µg PaExoA into the right middle ear affected the median DPOAE thresholds. As a control we used 20 µl NaCl solution injected into the left middle ears, which showed no significant rise in DPOAE thresholds (Paper IV, Fig. 1).

Intratympanic injection of 15 µg PaExoA (group 1) induced an increase in median DPOAE thresholds for low-frequency region by 15, 22 and 13 dB SPL at 1, 2 and 3 hours after exotoxin injection. The median DPOAE value was the same as control at 4 hours after injection, but rose again by 12 dB SPL from control value 5 hours after injection. For the medium frequency region the rise in median DPOAE thresholds was 13, 22 and 5 dB SPL at 1, 2 and 3 hours after PaExoA injection. There was no change in the median DPOAE value at the 4-hour measurement, compared with control, but 5 hours after injection the median value had risen by 8 dB SPL. For the high-frequency region the median DPOAE thresholds rose by 28, 29, 15, 11 and 8 dB SPL at 1, 2, 3, 4 and 5 hours after PaExoA injection (Paper IV, Fig. 2).

Intratympanic injection of 20 µg PaExoA (group 2) significantly raised the median DPOAE thresholds for all frequency regions as follows: for the low-frequency region the median DPOAE thresholds had risen by 13, 10, 16, 10, 16 and 37 dB SPL at ½, 1, 2, 3, 4 and 24 hours after PaExoA injection respectively. For the medium frequency region the median DPOAE values rose by 9, 9, 14, 17, 24 and 36 dB SPL and for the high-frequency region they rose by 11, 16, 5, 5, 13 and 44 dB SPL at ½, 1, 2, 3, 4 and 24 hours after PaExoA injection respectively (Paper IV, Fig. 3).

5. Effect of Edaravone on DPOAE changes induced by PaExoA (Paper IV)

Injection of 20 µg PaExoA and then 3 mg/kg Edaravone 1, 2 and 4 hours later had no evident effect on the median DPOAE threshold increases induced by exotoxin (groups A, B and C). The only exception was a very limited effect on the rise in median DPOAE thresholds for the high-frequency regions.
In group A the median DPOAE thresholds for the low-frequency region rose by 16, 13 and 13 dB SPL from the control value at 2, 4 and 24 hours respectively, after Edaravone injection. For the medium frequency region the values rose by 11, 8 and 17 dB SPL, and for the high-frequency region, by 8, 2 and 12 dB SPL at 2, 4 and 24 hours respectively after Edaravone injection.

In group B the median DPOAE thresholds for the low-frequency region rose by 15, 20 and 23 dB SPL from the control value at 2, 4 and 24 hours respectively after Edaravone injection. For the medium frequency region the values rose by 15, 10 and 15 dB SPL, and for the high-frequency region the increase was only 5, 4 and 3 dB SPL at 2, 4 and 24 hours respectively after Edaravone injection.

In group C for the low-frequency region the median DPOAE values rose by 12, 12 and 43 dB SPL, and for the medium frequency region, only 7, 6 and 46 dB SPL at 2, 4 and 24 hours respectively after Edaravone. For the high-frequency region the median DPOAE thresholds declined by 2 and 1 dB SPL at 2 and 4 hours after Edaravone injection, but rose by 38 dB SPL 24 hours after Edaravone injection, as for the low and medium frequency regions (Paper IV, Fig. 4).
Discussion

Hearing loss or ABR threshold shift changes after tobramycin exposure (Paper I)

In the cochlea, aminoglycosides preferentially damage the OHCs, leaving supporting cells and IHCs less affected. Differing vulnerability to the free radicals appears to be one explanation; there are several reasons. One may be that OHCs have a lower endogenous intracellular antioxidant ability than IHCs, due to their lower glutathione concentration [46]. Glutathione levels vary from the base to the apex of the cochlea [47]. Another explanation could be an unequal activation of the nuclear factor kappa B, which contributes to ROS-induced cell signaling [48].

Dormans et al. [49] investigated the ototoxicity of tobramycin in the rat using doses of 10-160 mg/kg. Those doses caused focal lesions restricted mainly to the basal turn of the cochlea with a dose-dependent extent of damage. Microscopically those focal lesions appeared as shortened, disordered or absent stereocilia in OHCs surface.

In our study tobramycin ototoxicity was demonstrated as a deterioration of ABR threshold shifts over time in two groups. The use of 160 mg/kg tobramycin cause an elevation in the ABR threshold shifts compared with the controls. In one group, ABR deterioration started 7 days after the first tobramycin injection, gradually increasing to reach the highest level by the end of the study (day 35). In the other group the deterioration was less pronounced, and started only 14 days after the first tobramycin injection.

We found a hearing loss pattern similar to that in Dormans’ study but with individual variations, which might be explained by the genetic variations in the outbred rats, or by the injection technique used. This technique, although already in clinical use, has the disadvantage of possibly being unable to maintain the inoculated substance concentration in the RWM niche at the same level for all the animals tested.

Effect of Edaravone on ABR threshold shifts (Paper I)

Aminoglycosides, cisplatin, noise and toxins all cause SNHL, possibly because all these factors increase oxidative stress, which can be a common pathway leading to hair cell death. Edaravone and other antioxidants could
be useful otoprotectants. Maetani et al. investigated the safety of Edaravone treatment in gerbils; their study showed no inner ear damage after intravenous injection [39].

In our study intravenous injection of Edaravone did not affect ABR threshold shifts, which makes us believe that it is a safe substance to use in inner ear research.

**Effect of Edaravone on tobramycin-induced hearing loss used at different times (Paper I)**

Free radicals are important agents in the pathogenesis of a variety of diseases. They are therefore an attractive subject for therapeutic intervention in those diseases. Edaravone is a free radical scavenger capable of trapping several types of free radicals. In numerous earlier studies on inner ear dysfunctions, Edaravone displayed otoprotective effects on barotrauma [43][50], acoustic trauma [51], noise-induced hearing loss [52] and streptomycin-induced vestibulotoxicity in guinea pig [44][53].

In the first part of our study, simultaneous intraperitoneal injection of Edaravone with tobramycin prevented the worsening of ABR threshold shifts and thus prevented tobramycin-induced hearing loss. Comparison of the mean ABR threshold shifts in rats given tobramycin and Edaravone simultaneously, versus those given tobramycin only, revealed significant differences in hearing between the protected and non-protected groups.

Surprisingly, the first significant differences appeared at low frequencies (2-6 kHz) on days 7 and 14 after the first injection. The hearing loss at low frequencies was less pronounced than at the high frequencies, but more stable, which produces earlier statistical significance. On days 28 and 35, the ABR threshold shift decline at high frequencies (14-20 kHz) predominated, revealing significant differences between the protected and non-protected groups.

The next question of interest was to establish the optimal time point for Edaravone injection which will still provide good otoprotection against tobramycin-induced hearing loss. In part two of this study we tried to answer this question, by testing if Edaravone given a few days after tobramycin could still be as protective as when we used it simultaneously with tobramycin. Knowing from part one of our study that tobramycin-induced hearing loss starts around day 14, we postponed Edaravone injections until days 7 and 8, and found that hearing protection was as good as in the group given Edaravone and tobramycin simultaneously. The other important finding was that giving only two Edaravone injections, on
days 7 and 8, had the same protective potential as when 10 Edaravone injections were given, in part one.

When Edaravone injections were postponed to days 14 and 15, only a temporary effect could be noted, suggesting that between day 7 and day 14 the oxidative stress became established and that it was too late for the scavenging capacity to rescue the hearing function irrevocably.

However, there was temporary protection on days 21 and 28, showing that this injurious process was still proceeding and possible to counteract. This suggests that in the clinical situation, even late intervention can halt ototoxicity to some extent.

Changes in ABR threshold shifts after PaExoA exposure (Control groups, Papers II and III)

Intratympanically injected PaExoA reached the inner ear by traversing the RWM, causing cochlear dysfunction. When this effect on the cochlea was investigated both morphologically and electrophysiologically, there was either reversible or irreversible hearing loss [11][12].

Initially, intratympanic application of PaExoA causes a combination of conductive and sensorineural hearing loss. The former has been regarded as reversible and of little importance [45]. This was confirmed in previous studies by analysing ABR latency/intensity curves. The sensorineural component was predominant in latency versus intensity curves after 14 days, while the conductive component was observed distinctly on day 5 after PaExoA injection [31].

Any signs of OM had completely disappeared by day 14 after instillation, suggesting that the hearing loss after 2 weeks was a pure SNHL.

In our studies we investigated the changes in ABR threshold shifts after various doses of PaExoA injected intratympanically into the middle ear.

In the first study we tested the effect of increasing doses 1, 2 and 10 µg PaExoA on hearing by measuring ABR thresholds. We observed that an increase in ABR threshold shifts occurred at all concentrations, but the measured shifts were still reversible.

Increasing the dose of PaExoA to 10 µg did not cause a more serious hearing loss than 1 µg only. This absence of difference may have been due to the limited numbers of animals in our groups, or to genetic differences in outbred rats. Small amounts of toxin might have escaped from the middle ear through the Eustachian tube, or initially been prevented from reaching the inner ear through the RWM, due to anatomical variations in the niche [54]. However, the course of the hearing loss was longer in the animals exposed to 10 µg, though still not permanent. This finding may have been due to the
ability of the inner ear to recover following exposure to PaExoA, or that the PaExoA dosage was still insufficient to cause an irreversible or permanent ABR threshold shift.

For these reasons, we attempted in the second study to identify a single dose of PaExoA that would induce a permanent ABR threshold shift and hence, irreversible SNHL. At the end of the study (day 35), when higher doses of 15 µg, 20 µg and 25 µg PaExoA were used, the animals sustained the desired permanent ABR threshold shift. We concluded that 15µg PaExoA was the lowest dose that could cause such a shift, measured 35 days after intratympanic endotoxin injection.

When the mean ABR threshold shift in the group given 15 µg PaExoA was compared at different times with group given 20 µg PaExoA, it was obvious that the shift was more significant in the group given the higher dose.

**Hearing after BDNF as otoprotectant from PaExoA ototoxicity (Papers II and III)**

In our first study we found that 4 µg BDNF intratympanically instilled simultaneous with various doses of PaExoA ranging from 1 to 10 µg produced a significant otoprotective effect by preventing ABR threshold shifts at almost all measured frequencies, except 4 and 12 kHz on day 2. This BDNF dose evidently prevented SNHL caused by much higher doses of PaExoA than used in a previous study [31]. BDNF safety was tested by intratympanic injection of 4 µg into the middle ear of 3 rats, whereupon the ABR thresholds showed that this dose of BDNF lacked ototoxicity.

The mechanism by which BDNF prevents hearing loss is not known but may involve suppression of the nitric oxide-reactive oxygen species (NO-ROS) cascade [55], probably by activating tyrokinase receptors expressed in many neuronal cells as well as in supporting cells of the inner ear.

Most probably, by passing the RWM, BDNF activates these receptors present in most neuronal tissues in the organ of Corti [56]. Similarly, a protective effect of BDNF has been found in other animal studies [57].

The passage of BDNF through the RWM could also be facilitated by PaExoA and consequently its protective effect would be more evident when intended for inner ear infections than for sudden deafness, because of the possible “pore-making” effect of the simultaneously injected exotoxin [58]. However, it is still debatable whether a thickened and inflamed RWM in COM facilitates or impairs the passage of particles from the middle to the inner ear [59].
In the second study on BDNF we investigated the otoprotective effect of 4 µg BDNF 12 and 72 hours after intratympanic instillation of 15 µg PaExoA.

We found that animals given 4 µg BDNF 12 hours after PaExoA showed ABR threshold shifts on days 7 and 14. These shifts were most prominent at 12, 14, 16 and 20 kHz (high-tone frequencies), but were less evident at the low-tone frequencies 2, 4, 6, 8 and 10 kHz.

This finding suggests that PaExoA had a greater toxic effect on the basal turn of the cochlea due to its proximity to the round window niche, i.e. the point of injection into the middle ear. The ABR threshold shifts diminished on days 21 and 28 to a range of 0 up to 4 dB SPL, while on day 35 they returned to the same levels as at control, for all frequencies except 12 kHz. This shows that even with a 12-hour delay we can still achieve otoprotection with a single dose of BDNF.

When 4 µg BDNF was injected with an interval of 72 hours after PaExoA, there were still significant changes in mean ABR thresholds on day 35 (end of the study), suggesting absence of protective effect from BDNF.

Changes in DPOAE thresholds after NaCl and tympanic membrane perforation (Paper IV)

Registration of DPOAEs before and then after only perforating the tympanic membrane with a Metal Tip® (0.4 x 60 mm) needle under a microscope, failed to show any significant change in the measured thresholds.

Injection of 20 µl NaCl in 13 left middle ears, followed by measurement of DPOAE thresholds after ½, 1-2 and 3-4 hours after instillation also failed to show any significant change in the thresholds.

These two findings confirm that a tiny perforation in the tympanic membrane and/or the presence of 20 µl NaCl solution in the rat middle ear does not significantly affect DPOAE thresholds.

The same result was found in a previous study on guinea pig (Ueda et al. 1998). They demonstrated that OAE levels did not alter significantly after half the volume of the bulla has been filled with fluid. In that study a small perforation of the tympanic membrane caused a loss of OAE, restricted to the lower frequencies; larger perforations caused a loss of OAE at the higher frequencies too [60].
Changes in DPOAE thresholds after PaExoA exposure (Paper IV)

DPOAEs test frequency-specific regions of the cochlea and are therefore regarded as particularly well suited for monitoring ototoxic injury. DPOAEs are easy to measure in small experimental animals and are less sensitive than transient evoked otoacoustic emissions (TEOAEs) to perforation of the tympanic membrane.

DPOAE analysis is considered suitable for studies on acquired hearing loss in which primarily the sensory hair cells are affected. For all these reasons we decided to study the very early effect of PaExoA on DPOAEs in rats.

To our knowledge, this is the first study utilizing DPOAEs to reveal PaExoA-induced inner ear dysfunction, as in all our previous studies, measurement of ABR threshold shifts was used to evaluate *P. aeruginosa* ototoxicity.

Intratympanically injected 15-20 µg PaExoA showed a significant increase in DPOAE thresholds within hours at different time points and different frequency regions tested. Although DPOAE is generated exclusively by OHCs which do not themselves activate primary auditory nerve fibers, a close relationship nevertheless exists between changes in OAE and hearing loss. Thus this early rise in DPOAE thresholds elicited by PaExoA suggests a possible ototoxic effect in the inner ear or SNHL, appearing within hours after intratympanic injection of an endotoxin.

In 8 rats we measured DPOAE thresholds 24 hours after PaExoA injection; at this time the rise in median DPOAE thresholds was higher when compared with measurements made ½, 1, 2, 3 and 4 hours after endotoxin injection. This suggests that the changes in DPOAE thresholds could persist for a long time and may be similar to the changes seen in ABR threshold shifts in our previous studies (Papers II and III). We did not continue measuring DPOAEs as the purpose of our study was to investigate the very early effects of PaExoA on DPOAEs.

Effect of Edaravone on PaExoA- induced increases in DPOAEs (Paper IV)

Takumida and Anniko showed that when injected simultaneously, whether intratympanically or intravenously, Edaravone prevented PaExoA-induced ABR threshold shifts. The same applied when Edaravone was injected intratympanically one hour after PaExoA [61]. In our study we investigated the effect of intratympanically injected Edaravone on the increases in PaExoA- induced DPOAE thresholds.
Edaravone was applied intratympanically in three different groups 1, 2, and 4 hours after 20 µg PaExoA, while DPOAE was measured on 2, 4, and 24 hours after Edaravone injection. When the median DPOAE thresholds at control were compared with those after Edaravone injection at the three time points stated above, there was no effect of Edaravone on the elevated DPOAE thresholds for the low and medium frequency regions.

There was only a limited increase in the median DPOAE thresholds for the high-frequency regions in the first two groups (Edaravone injected 1 and 2 hours after PaExoA respectively) but no change in the third group (Edaravone applied 4 hours after PaExoA) this result mean that intratympanically injected Edaravone may limits the PaExoA-induced DPOAE threshold increase in the high-frequency region.
General conclusion

Paper I: Edaravone is an effective protective agent against tobramycin-induced ototoxicity, when administered simultaneously. Administration delayed to 7 days still affords complete protection, whereas delay to day 14 appears to weaken its prophylactive effect.

Paper II: Intrinsically instilled 4 µg/ 20µl BDNF in the middle ear of rats is not ototoxic. Intratympanical injection of increasing doses PaExoA caused a reversible ABR threshold shift. Simultaneous intratympanic instillation of 4 µg BDNF with any dose PaExoA (1µg, 2µg or 10µg) will prevent such ABR threshold shifts, and have a significant otoprotective effect on PaExoA.

Paper III: Intratympanic instillation of 15µg, 20µg or 25µg PaExoA caused a permanent ABR threshold shift, sustained significantly until the end of the study (day 35). A single dose of 4 µg BDNF, applied intratympanically delayed 12 hours after a 15 µg PaExoA injection, will still achieve a significant protective effect against exotoxin-induced ABR threshold shifts. However, if the delay in BDNF injection is increased to 72 hours, no otoprotective effect is observed.

Paper IV: Intratympanic injection of 15 µg or 20 µg PaExoA induced a significant rise in DPOAE thresholds within hours after instillation. This finding suggests that PaExoA reaches the inner ear much quicker than known from previous studies. Neither tiny perforation of the tympanic membrane nor 20 µl of NaCl solution affected the DPOAE thresholds significantly.

Edaravone instilled intratympanically 1, 2 and 4 hours after 20 µg PaExoA, failed to show any effect on DPOAE threshold increase for the low- and medium frequency regions. There was a limited effect of Edaravone on the exotoxin-induced DPOAE threshold increase for the high-frequency region.
Sammanfattning på svenska

Bakgrund

Hörselnedsättning är fortfarande en folksjukdom. Frånsett ålderspåverkan så är förvärvad hörselnedsättning den näst vanligaste orsaken till dålig hörsel, t ex kronisk otit eller farmakoterapi med antibiotika av aminoglykosidtyp eller behandling med cellgifter som cisplatin.


Antibiotika är för närvarande den vanligaste behandlingen vid otit medan kirurgi kan bli aktuellt vid behandling av otitkomplikationer. Hörapparater i första hand och ibland cochleaimplantat (CI) används för att ge patienter med SNHN en bättre hörsel.

Behovet att hitta alternativa behandlingsmetoder har blivit mer aktuellt senaste åren, på grund av ökad bakteriell antibiotikaresistens samt att det är svårt och dyrt att finna och producera nya antibiotika. Under de senaste åren många forskare runt om i värden letat efter substanser (s.k. hörselskyddande substanser, otoprotectants) som kan hindra t ex endotoxiner eller aminoglycosider att skada innerörat och orsaka SNHN.
I våra studier har vi använd Brain-Derived Neurotrophic Factor (BDNF) och Edaravone med potentiellt hörselskyddande effekt. BDNF finns tidigt under embryonalutvecklingen i innerörat och spelar en viktig roll såväl under fostertiden som senare under livet för att bibehålla normal innerörefunktion. Tidigare studier har visat att BDNF kan skydda innerörat mot cisplatin-inducerad skada samt mot bullerskada. I våra studier undersökte vi om BDNF kan skydda innerörat från skador orsakade av PaExoA och om hur längre kan vi avvakta efter toxinexposition med att tillföra BDNF för att fortfarande ge en hörselskyddande effekt.

Edaravone (MCI-186,3-methyl-1-phenyl-pyrazoline-5-one) är en free radical scavenger som sedan några år tillbaka i Japan används kliniskt för att minska ischemiska hjärnskador, behandling av akuta hjärtinfarkter och rheumatoid artrit. Substansen tycks således ha en avsevärd bredd i sin terapeutiska arsenal och kan därför tänkas vara en form av allmänt skyddande substans på cellbiologisk nivå. Vi undersökte om Edaravone kan klassas som en mer allmän typ av otoprotectant och hindra uppkomsten av inneröra skada orsakad av såväl tobramycin som av PaExoA.

Hjärnstamsaudiometri (ABR) och Distortion Product Otoacoustic Emissions (DPOAE) används båda för en objektiv analys av hörselnäckans funktion och för att kunna fastställa hörtrösklar.

ABR (2, 4, 6, 8, 10, 12, 14, 16 respektive 20 kHz) är av typen evoked response vilken innebär att man först stimulerar örat och sedan direkt registrerar ett elektrofysiologiskt svar på olika centrala nivåer (hörselnerven, hjärnstam, hjärna) av hörselimpulssvar. ABR kräver ingen annan medverkan än att den som undersöks ligger tyst och undviker rörelse. Försöksdjur måste sövas.

Vid DPOAE registreras de yttre hårcellernas aktivitet som svar på utifrån kommande ljudstimuli. Man använder en mätstation (probe) i hörselgångens mynning. I proben finns små högtalare, som spolar upp ljudstimuli vilka sätter i gång vibrationerna i innerörat, samt en mikrofon som registrerar det ljud som alstras av de yttre hårcellerna. Om innerörat stimuleras samtidigt med två toner som ligger nära varandra i frekvens genererar hörselnäckan en tredje ton som kallas för distortion product. Det är därför möjligt att registrera OAE samtidigt som man stimulerar innerörat med olika frekvenser.
Arbete 1: Protective effect of Edaravone against Tobramycin-induced Ototoxicity.

Frågeställning: kan Edaravone förhindra uppkomsten av hörselskada vid exposition för tobramycin?

Den första delen av arbetet 1 (n=21 albino Sprague-Dawley råttor) uppdelades i tre grupper. Grupp A (n= 2) fick 3mg/Kg Edaravone intravenös. Grupp B (n= 8) gavs tio subkutana injektioner tobramycin (160mg/kg) + 0,3 ml koksalt som i.p. injektion under 2 veckor. Grupp C (n=11) tio subkutana injektioner tobramycin (160 mg/kg) + tio i.p. injektioner Edaravone (3 mg/kg) samtidigt.

I andra delen av arbete 1 använde vi (n=13) uppdelad i tre grupper; Grupp D (n=4) fick tio subkutana injektioner tobramycin (160mg/kg) + 0,3 ml koksalt i.p. under 2 veckor. Grupp E (n=5) tio subkutana injektioner tobramycin (160mg/kg) + två i.p. injektioner Edaravone (3 mg/kg) 7 respektive 8 dagar efter första tobramycin injektionen. Grupp F (n=4) tio subkutana injektioner tobramycin (160mg/kg) + två i.p. injektioner Edaravone (3 mg/kg) 14 respektive 15 dagar efter första tobramycin injektionen.

ABR används för att bedöma hörseln. Före varje ABR-mätning undersöktes mellanöronen med mikroskop för att verifiera trumhinne- och mellanörestatus. ABR mätning utfärdas före och 3, 7, 14, 21, 28 och 35 dagar efter tobramycin exposition.

Slutsatser arbete 1:

1. Alla djur som exponerats för enbart tobramycin eller tobramycin + koksalt uppvissade försämring av ABR threshold shifts, dvs hörselskada uppmättas;
2. När tobramycin och Edaravone anbringades samtidigt uppkom ingen hörselskada;
3. Tillförsel av Edaravone 1 vecka efter exposition för tobramycin gav fortfarande en skyddande effekt som kvarstod efter 35 dagar (studiens slutdag);
4. Tillförsel av Edaravone 2 veckor efter exposition för tobramycin gav en partiell men övergående skyddande effekt.
Arbete 2: BDNF as Otoprotectant in Toxin-induced Hearing Loss.

Frågeställning: kan samtidigt tillförsel av BDNF intratympanalt förebygga försämring av hörtröskeln om man successivt ökar koncentrationen av PaExoA?

I arbete 2 (n=33 albino Sprague-Dawley råttor) uppdelades i fem kontrollgrupper och tre behandlingsgrupper.

Kontrollgrupper (n=20) enligt följande: Grupp A (n=4) fick intratympanal injektion av koksalt. Grupp B (n=3) 4 µg BDNF intratympanalt. Grupp C (n=4) 1 µg PaExoA intratympanalt. Grupp D (n=4) 2 µg PaExoA. Grupp E (n=5) 10 µg PaExoA.

Behandlingsgrupper (n=13) enligt följande: Grupp 1 (n=3) fick 1 µg PaExoA + 4 µg BDNF samtidigt intratympanalt. Grupp 2 (n=5) 2 µg PaExoA + 4 µg BDNF. Grupp 3 (n=5) 10 µg PaExoA + 4 µg BDNF.

ABR används för att bedöma hörseln. Före varje ABR-mätning undersöktes mellanöronen med mikroskop. ABR-mätning gjordes före respektive 2, 5, 15 och 21 dagar efter intratympanala injektioner.

Slutsatser arbete 2:

1. 4 µg BDNF anbringades intratympanalt orsakade inga förändringar i ABR threshold shifts.
2. Olika doser av PaExoA orsakade förändringar i ABR threshold shifts (hörselnedsättning).
3. 4 µg BDNF tillförd samtidigt med PaExoA förebygger förändringar i ABR threshold shifts oavsett koncentrationen av exotoxinet.
Arbete 3: Early Hearing Protection by Brain –Derived Neurotrophic Factor.

Frågeställning: (a) vilken dos PaExoA orsakar en permanent ABR threshold shifts vid intratympanalt applikation? (b) Kan tillförsel av 4 µg BDNF intratympanalt inom 12-72 timmar efter exposition för PaExoA ge samma grad av hörselskyddande effekt som när 4 µg BDNF applicerades samtidigt med PaExoA?

I arbete 3 (n=28 albino Sprague-Dawley råttor) uppdelades i tre kontroll- och två behandlingsgrupper.

Kontrollgrupper (n=16) enligt följande: Grupp1 (n=8) fick 15 µg PaExoA intratympanalt. Grupp 2 (n=5) 20 µg PaExoA. Grupp 3 (n=3) 25 µg PaExoA.

Behandlingsgrupper (n=12): Grupp A (n=6) fick 15µg PaExoA + 4 µg BDNF intratympanalt (efter 12 timmar). Grupp B (n=6) 15 µg PaExoA + 4 µg BDNF (efter 72 timmar).

ABR används för att bedöma hörseln. Före varje ABR-mätning undersöktes mellanöronen med mikroskop. ABR- mätning utfärdas före och 7, 14, 21, 28 och 35 dagar efter exposition för PaExoA.

Slutsatser arbete 3:

1. PaExoA kan ge en permanent hörselskada vid en dos av 15 µg.
2. Graden av förändringen i ABR threshold shifts (hörselskadan) är dosberoende.
3. 4 µg BDNF skyddar hörseln om BDNF tillförs 12 timmar efter exposition för PaExoA men inte efter 72 timmar.
Arbete 4: Temporary Dysfunction of Outer Hair Cells after Intratympanic Application of Pseudomonas aeruginosa Exotoxin A.

Frågeställning: (a) När uppkommer tidigast en förändring i DPOAE thresholds efter intratympanal injektion av PaExoA? 
(b) Kan intratympanal injektion av Edaravone förhindra detta?

I arbete 4 (n=34 hankön albino Sprague-Dawley råttor) uppdelas i två undergrupper.

Den första undergruppen (n=20) analyserades enligt följande:

Grupp 1 (n=9) fick 15 µg PaExoA anbringat i höger mellanöra. Koksalt 20 µl som kontroll i vänster mellanöra (n=6). DPOAEs av höger öron registrerats före exposition (kontroll värde) och därefter 1 timme (n=7), 2 timmar (n=6), 3 timmar (n=7), 4 timmar (n=4) och 5 timmar (n=2). DPOAEs av vänster öron registrerades (n=6) före respektive efter 1-2 timmar (n=4) och 3-4 timmar (n=6).

Grupp 2 (n=11) 20 µg PaExoA anbringat i höger mellanöra, koksalt 20 µl som kontroll i vänster mellanöra (n=7). DPOAEs av alla höger öron mättes före respektive 30 minuter, 1, 2, 3, 4 och 24 timmar (n=8) efter exponering för PaExoA. DPOAEs av vänster öron mättes före respektive 30 minuter efter koksalt injektion (n=7).

I andra undergruppen (n=14) analyserades enligt följande:

Grupp A (n=4) fick 20 µg PaExoA anbringat i höger mellanöra + 3mg/kg Edaravone intratymponalt (efter 1 timme). Grupp B (n=6) 20 µg PaExoA anbringat i höger mellanöra + 3mg/kg Edaravone intratymponalt (efter 2 timmar). Grupp C (n=4) 20 µg PaExoA anbringat i höger mellanöra + 3mg/kg Edaravone intratymponalt (efter 4 timmar). DPOAEs av alla höger öron mättes före PaExoA-exposition (kontroll), och därefter 2, 4 och 24 timmar efter appliceringen av Edaravone.

Slutsatser arbete 4:

1. PaExoA förorsakar tidiga förändringar av DPOAE thresholds dvs. påverkar hörselsnäckans funktion inom 0,5-2 timmar.
2. Koksalt påverkar inte DPOAE thresholds.
3. Edaravone kunde inte förebygga DPOAE förändringar orsakade av 20 µg PaExoA.
Sammanfattningen av alla arbeten:

1. Edaravone fungerar som hörselskyddande substans och kan skydda mot sensorineural hörselnedsättning orsakad av tobramycin om Edaravone tillförs inom en vecka efter att behandling med tobramycin startat.

2. BDNF kan förebygga SNHN om hörselnäckan exponeras med totaldoser av PaExoA upp till 15 µg PaExoA men måste tillföras inom 12 timmar.

3. PaExoA kan ge en permanent hörselskada vid en dos av 15 µg och graden av hörtröskelförsämring är dosberoende.

4. PaExoA påverkar DPOAE thresholds inom 0,5-2 timmar som innebär att yttre hårcellsfunktion kan påverkas ytterst snabbt vilket man tidigare ej kunnat påvisa med några andra elektrofysiologiska mätmetoder.

5. Edaravone har en begränsad hörselskyddande effekt vid höga koncentrationer av PaExoA.
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// Adnan
References


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