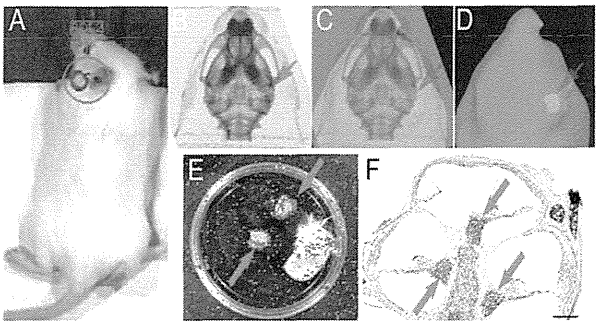

Figure 1. Strong bioluminescence around the ear of GFAP-luc derived from cochlear spiral ganglion. Previous bioimaging study with IVIS reported luminescence around the ear of GFAP-luc. We first reveal here that the signal results from spiral ganglion. (A) Signal observed after local injection of luciferin via tympanic membrane. (B–D) Bioluminescence image of mouse head overlaid with a corresponding micro-CT image. (B) Micro-CT image of the head. (C) Merged image of A and C. (D) Photon bioluminescence image of the head. Note the signal was overlapped with temporal bone and not with superficial skins. (E) Ex-vivo bioluminescence in a freshly dissected temporal bone. Strong signal in the cochleae was observed (arrows), while no signal was detected in the skin and ear cartridge (right). (F) Immnohistochemistry for luciferase in the cochlea showed the expression was high in spiral ganglion (arrows).
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associated with using microdialysis to analyze the pharmacokinetics of the inner: (1) technical difficulty of the measurement; (2) restricted locations available for measurement (i.e., basal turn of cochlea); (3) inability to quantify clearance values accurately for the inner ear; and (4) inability to measure the time course in the same animal. To solve these previously reported problems, we developed a new *in vivo* imaging technique and investigated whether RW obstructions block drug delivery into the inner ear.

Materials and Methods

GFAP-Luc transgenic mice

The transgenic GFAP-luc mice (FVB/N background; [5]) were obtained from Xenogen Corporation (Alameda, CA), and were backcrossed with CD1 (ICR, SLC Japan) for 5 or 6 generation. The mice contain a firefly luciferase gene expression cassette that is regulated by 12 kb of the murine GFAP promoter and the human β-globin intron 2 [5]. Luciferin delivered into the inner ear of these mice reacts with luciferase that is expressed in the GFAP-expressing cells in the cochlear nerve and spiral ganglion, and the resulting photons are detected by the camera. All experiments were approved by the Animal Care and Use Committee of Keio University (Permit Number: 08020) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health, Bethesda,MD). All surgery was performed under ketamine and xylazine anesthesia, and all efforts were made to minimize suffering.

We divided the GFAP-Luc mice into three groups: intraperitoneal (i.p) injection group (N = 6), RW niche obstruction with transtympanic injection group (N = 3), and non-obstruction with transtympanic injection group (N = 8). The wild type mice group was transgene negative littermates (N = 2).

Round window niche obstruction surgery

Adult GFAP-Luc mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). As previously described, an incision was made from the left post-auricular region to the submandibular region, and the tympanic bulla was exposed without damaging the facial nerve and vessels [6]. The bulla was perforated with a surgical needle, and the hole was expanded with forceps to approximately 2 mm diameter in order to cover over the RW with fascia (RW obstruction) without disturbing the auditory ossicular chain, mimicking clinical cases with RW false membrane. The hole in the cochlea and the opening in the tympanic bulla were sealed with carboxylate cement. Muscle and skin were sutured. It took approximately 20 min to complete this procedure. Left earlap was surgically removed to expose tympanic membrane for the injection of luciferin and to reduce the background.

Transtympanic or intraperitoneal injections

Just prior to imaging, we injected 0.1 ml or 0.5 ml of luciferin substrate (150 mg/kg) directly onto the round window membrane or intraperitoneally, respectively. Transtympanic injection was performed securely under the surgical microscope.

Statistics

Peak bioluminescence intensity and the time when the luminescence was maximum in individual groups (i.e., ip, TT injection and TT injection after round window closure) was examined by non-parametric t-test. All scores were averaged and analyzed with SPSS software 19.0 (Chicago, USA).

Bioluminescence imaging

An IVIS spectrum and CCD optical macroscopic imaging system (Xenogen, Alameda, CA) was used for spatiotemporal detection of the luciferase - luciferin reaction. *In vivo* bioluminescent images were captured immediately after i.p. injection (0.5 ml) or after transtympanic injection (TT, 0.1 ml) of D-luciferin (D-(−)-2-(6-hydroxy-2-benzothiazolyl) thiazone-4-carboxylic acid). The field-of-view was set at 10 cm. The photon count was analyzed between 0 and 40 min after the i.p. and TT injections of luciferin substrate. The integration time was fixed at 5 min for each image. All images were analyzed with Living Image software (Xenogen, Alameda, CA). The optical signal intensity was expressed as photon flux (photon count), in units of photons/s/cm^2/steradian. Each image was displayed as a pseudocolored photon-count image superimposed onto a grayscale anatomic image. To quantify the measured light, we defined regions of interest (ROI) over the cell-implanted area and examined all values in the same ROI.

Immunohistochemistry

The animals were anesthetized with ketamine (100 mg/g) and xylazine (10 mg/g) and decapitated. The inner ear was removed and locally perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) followed by overnight fixation with the same fixative and wash with 0.1 M PBS. The otic capsules were embedded in paraffin and sectioned at 4 μm. For immunostaining, tissue sections were stained with anti-luciferase antibody (polyclonal goat, Promega) diluted at 1:75 for 60 min at room temperature, followed by an anti-goat secondary antibody (Histofine® Simple Stain MAX-PO, Nichirei Bioscience; 414351).

Results

Characterization of the cochlea of GFAP-luc mouse

Previous bioimaging of GFAP-luc showed a signal around the ear [5]. We first investigated the origin of the signal. The signal was observed even after the local injection of luciferin thru tympanic membrane in the mouse of which earlap had been removed and the signal was seen over the temporal area (Fig. 1A).

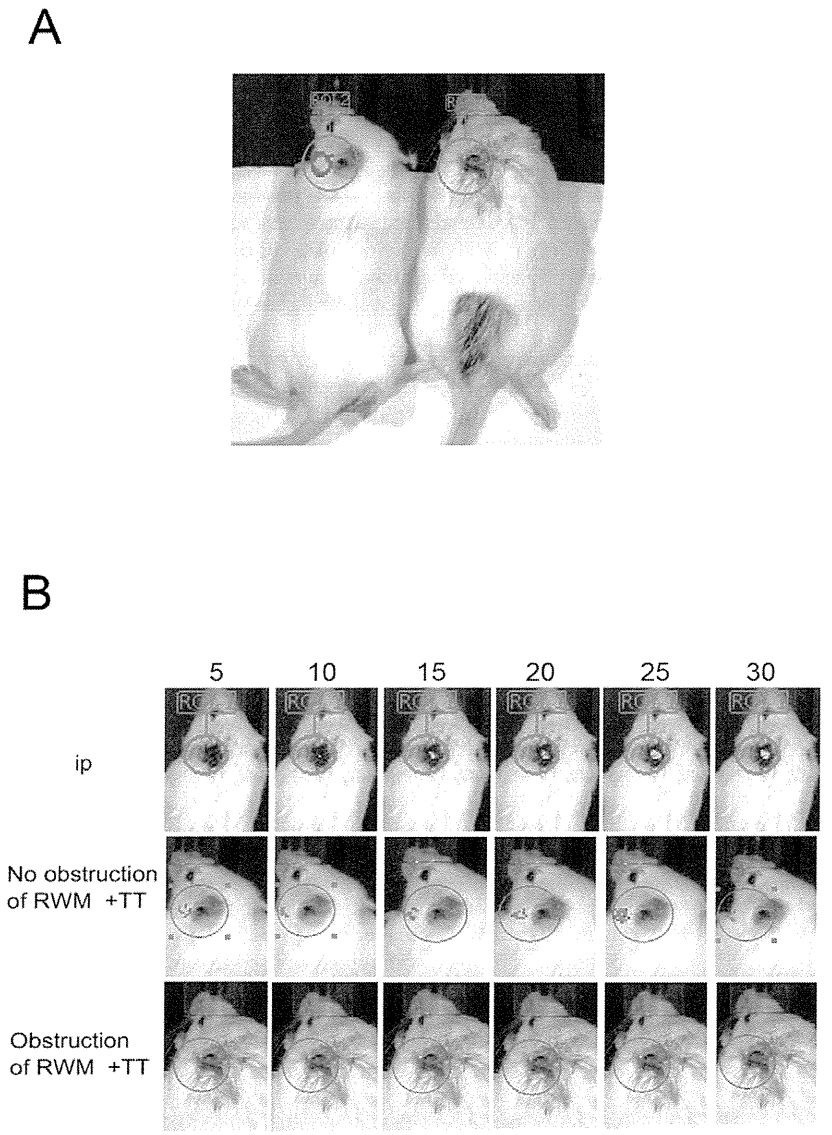

Figure 2. Photon bioluminescence in GFAP-luc mice. (A) Differences in photon bioluminescence of round windows with and without obstructions. The left panel shows photon bioluminescence in a mouse's left ear, in which the round window has been obstructed. The right panel shows the lack of bioluminescence in the mouse's right ear, in which the round window remains intact. (B) Time course showing photon bioluminescence in ears of mice with ip, with and without round window obstruction. ip;intraperitoneal injection, RWM; round window membrane.
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We then superimposed the biophotonic results over micro-CT image and the result clearly demonstrated that the signal was overlapped with temporal bone and not with superficial skins (Fig. 1B–D). We also dissected temporal bone of GFAP-luc and the luminescence of each part was measured: Strong signal was seen in the cochleae (Fig. 1E, arrows), while no signal was detected in the skin and ear cartridge (right). Collectively, we speculated that the signal in the ear area of the mice is derived from the cochlea. We then performed immnohistochemistry for luciferase in the cochlea, showing the expression was high in spiral ganglion (Fig. 1F, arrows). Together, we conclude that the bioluminescence in the area of GFAP-luc reflects the reaction of luciferase and luciferin in the cochlear spiral ganglion and thus we monitor the delivery of luciferin to the ganglion by measuring the biophotonic signals using IVIS.

Bioimaging

In the TT injection group of mice, photon bioluminescence was observed five minutes after injection, reaching a peak 15–20 minutes after injection (Fig. 2B). Bioluminescence intensity gradually faded thereafter. In the i.p. injection group of mice, detectable bioluminescence appeared much later than that in the TT injection group. As expected, no bioluminescence was observed in the RW obstruction group (negative control). The lack of reaction in the negative control indicated that luciferase did not reach the inner ear.

Time curves

Trends of bioluminescence in GFAP-luc mouse and wild type control in the individual groups were examined (Fig. 3A, mean ± SD). Peak bioluminescence intensity in the TT injection group was

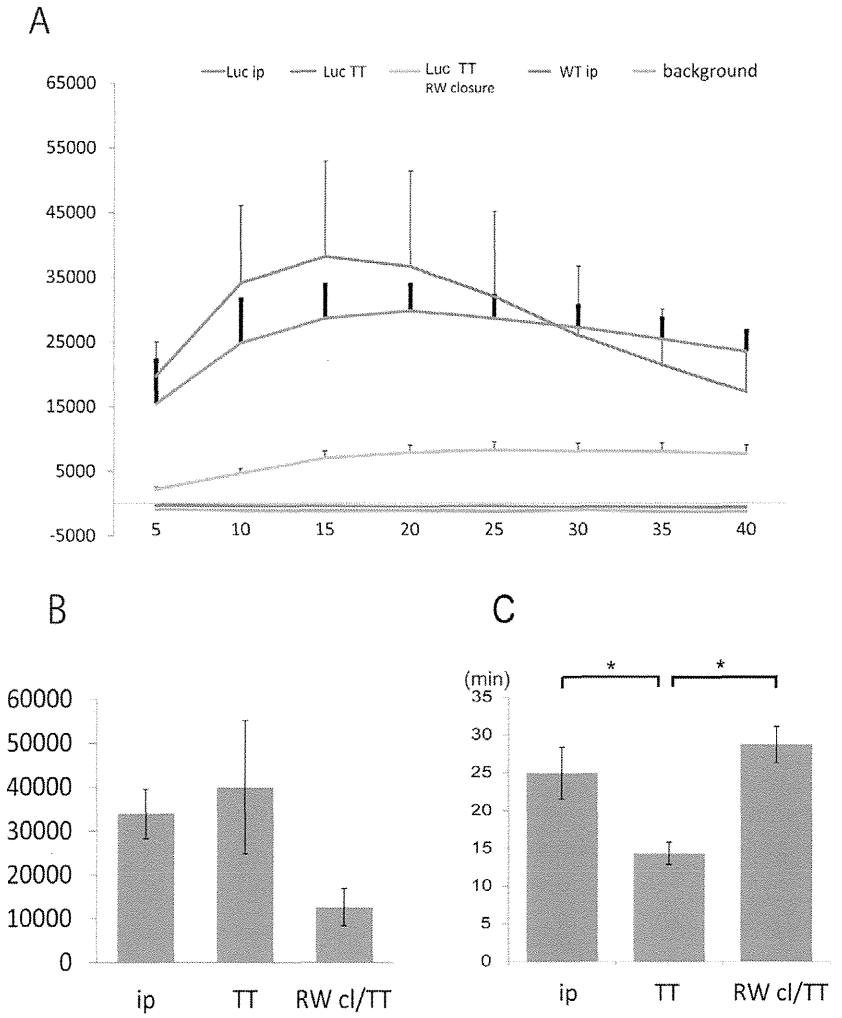

Figure 3. Time course of photon bioluminescence. (A) Time course of photon bioluminescence in five groups of animals: Luc ip, luciferase-expressing mice receiving intraperitoneal injections; Luc TT, luciferase-expressing mice receiving transtympanic injections; Luc TT RW closure, luciferase-expressing mice that received transtympanic injections after round window membrane obstruction; WT ip, wild-type mice receiving intraperitoneal injections. Background, background (no photons). (B) Bar graphs showing the highest photon counts by region. ip, intraperitoneal injection; TT, transtympanic injection; RW cl/TT, transtympanic injection after round window membrane obstruction. (C) Delivery time into GFAP-expressing cells of inner ear. Average time when the signal was maximum in each group was shown (mean ± SEM). Same conventions as in Fig. 2. *$p<0.05$.

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reached sooner after injection than in the i.p. injection group (Fig. 3C, mean ± SEM $p<0.05$). Time course of the photon emission in the individual animals and their peak of TT injection group are in Fig. 4A and 4B, respectably.

Discussion

We found that RW niche obstructions, such as false membranes or fibrous connective tissue membranes, are critical for drug delivery into the inner ear. Therefore, it is very important to examine the RW before injecting drugs into the inner ear [3].

The present study also assessed the time needed for TT injections of drug delivery system (DDS) to reach the inner ear and traced DDS distribution in the inner ear. A previous report demonstrated that one hour after TT injection of methylprednisolone, dexamethasone, and hydrocortisone, drug concentration in the perilymphatic space becomes elevated [7]. However, a few studies report shorter drug elevation times after injection [8,9,10]. In this study, we detected bioluminescence in the inner ear earlier than expected; five minutes after TT injection of luciferin. A study in guinea pigs showed that significantly higher dexamethasone levels were detected in the inner ear just 30 minutes after its transtympanic administration, gradually dropping to zero after 6 hours [9]. The time differences may be due to differences in the sizes of animals (guinea pigs vs. mice), and/or due to differences in drug metabolism and drug molecular size (steroid vs. luciferin).

We observed variations in bioluminescence intensity among animals in the TT group. Individual variations between animals, especially in the local injection group, have been reported previously [7,8]. We speculated that the injected luciferin goes to the Eustachian tube of animals.

In Chandrasekhar et al., drug concentration in perilymph was 5.52±2.80 μg/dL after intravenous (i.v.) injection of 0.45 mg/kg dexamethasone, but 13.2±10.6 μg/dL after location injection of

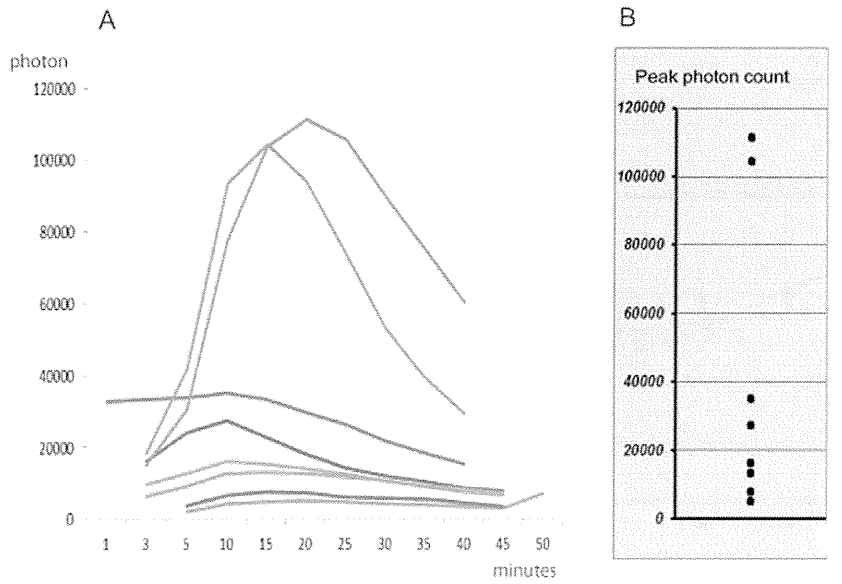

Figure 4. Quantification of photons in different groups of mice with TT injections. (A) Time course of photon bioluminescence for each group of mice. (B) Peak photon counts.
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10 mg/mL dexamethasone. Although the dose of the local injection was approximately 10 times higher than that of the systemic injection, drug concentration in perilymph was nearly two times lower (5.52±2.80 μg/dL vs. 13.2±10.6 μg/dL) [8]. In the present study, although the dose of the systemic injections was five times higher than that of TT injections, drug concentration in perilymph was almost the same or slightly higher.

Here, we were able to observe, in real time, drug delivery and drug distribution in animals *in vivo*, enabling us to determine the time course of drug delivery. One limitation of this study, however, is that we investigated the delivery of only luciferin into the inner ear, not drugs commonly used to treat inner ear disorders. Differences in the molecular weights of luciferin and steroids could affect delivery and diffusion time. One future application of this system is drug tagging, in which a drug linked to luciferin can be traced. This drug-tagging system may help physicians determine which drug can be delivered into which portion of the inner ear.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SK MF KO. Performed the experiments: SK MF SS. Analyzed the data: SK MF. Contributed reagents/materials/analysis tools: AY MN HJO HO. Wrote the paper: SK MF SS.

References

1. Rauch SD, Halpin CF, Antonelli PJ, Babu S, Carey JP, et al. (2011) Oral vs intratympanic corticosteroid therapy for idiopathic sudden sensorineural hearing loss: a randomized trial. JAMA: the journal of the American Medical Association 305: 2071–2079.
2. Silverstein H, Rowan PT, Olds MJ, Rosenberg SI (1997) Inner ear perfusion and the role of round window patency. The American journal of otology 18: 586–589.
3. Kanzaki S, Saito H, Inoue Y, Ogawa K (2012) A new device for delivering drugs into the inner ear: Otoendoscope with microcatheter. Auris, nasus, larynx 39: 208–211.
4. Thorne M, Salt AN, DeMott JE, Henson MM, Henson OW Jr, et al. (1999) Cochlear fluid space dimensions for six species derived from reconstructions of three-dimensional magnetic resonance images. The Laryngoscope 109: 1661–1668.
5. Zhu L, Ramboz S, Hewitt D, Boring L, Grass DS, et al. (2004) Non-invasive imaging of GFAP expression after neuronal damage in mice. Neuroscience letters 367: 210–212.
6. Iizuka T, Kanzaki S, Mochizuki H, Inoshita A, Narui Y, et al. (2008) Noninvasive in vivo delivery of transgene via adeno-associated virus into supporting cells of the neonatal mouse cochlea. Hum Gene Ther 19: 384–390.
7. Parnes LS, Sun AH, Freeman DJ (1999) Corticosteroid pharmacokinetics in the inner ear fluids: an animal study followed by clinical application. The Laryngoscope 109: 1–17.
8. Chandrasekhar SS, Rubinstein RY, Kwartler JA, Gatz M, Connelly PE, et al. (2000) Dexamethasone pharmacokinetics in the inner ear: comparison of route of administration and use of facilitating agents. Otolaryngology–head and neck surgery: official journal of American Academy of Otolaryngology-Head and Neck Surgery 122: 521–528.
9. Liu HJ, Dong MM, Chi FL (2006) Dexamethasone pharmacokinetics in Guinea pig inner ear perilymph. ORL; journal for oto-rhino-laryngology and its related specialties 68: 93–98.
10. Hahn H, Kammerer B, DiMauro A, Salt AN, Plontke SK (2006) Cochlear microdialysis for quantification of dexamethasone and fluorescein entry into scala tympani during round window administration. Hearing Research 212: 236–244.

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Protection Against Ischemic Cochlear Damage by Intratympanic Administration of AM-111

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Objective: AM-111, a cell-permeable peptide inhibitor of c-Jun N-terminal kinase, was investigated for its protective effects against ischemic damage of the cochlea in gerbils.
Methods: Transient cochlear ischemia was introduced in animals by occluding the bilateral vertebral arteries for 15 minutes. Then, 10 μl of AM-111 at a concentration of 1, 10, or 100 μM in hyaluronic acid gel formulation was applied onto the round window 30 minutes after the insult. Gel without active substance was used in a control group. Treatment effects were evaluated by auditory brainstem response (ABR) and histology of the inner ear.
Results: In controls, transient cochlear ischemia caused a 25.0 ± 5.0 dB increase in the ABR threshold at 8 kHz and a decrease of 13.3 ± 2.3% in inner hair cells at the basal turn on Day 7. Ischemic damage was mild at 2 and 4 kHz. When the animals were treated with AM-111 at 100 μM, cochlear damage was significantly reduced: the increase in ABR threshold was 3.3 ± 2.4 dB at 8 kHz, and the inner hair cell loss was 3.1 ± 0.6% at the basal turn on Day 7. The effects of AM-111 were concentration dependent: 100 μM was more effective than 1 or 10 μM.
Conclusion: Direct application of AM-111 in gel formulation on the round window was effective in preventing acute hearing loss because of transient cochlear ischemia. **Key Words:** AM-111—c-Jun N-terminal kinase—Mongolian gerbil—Prevention of hearing loss—Transient cochlear ischemia.

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Cochlear ischemia is considered to be one of the etiologic factors that can trigger idiopathic sudden sensorineural hearing loss (ISSNHL). We developed an animal model of transient cochlear ischemia in gerbils and studied the mechanisms of ischemia/reperfusion injury of the cochlea (1–3). In this model, acute sensorineural hearing loss was induced, mainly at higher frequencies. Histologic studies showed that the ischemic insult caused apoptotic cell death of the neural structures in the cochlea; the damage was more severe in the inner hair cells (IHCs) than in the outer hair cells (OHCs) (4,5). Using this animal model, we investigated a variety of candidate medicines for prevention of ischemia-induced hearing loss (6–10).

AM-111 is a cell-penetrating peptide that selectively inhibits the c-Jun N-terminal kinase (JNK) signaling pathway in the process of apoptotic cell death. It is prepared in a hyaluronic acid gel formulation to be placed on the round window membrane. When administered, it is transferred to virtually all sensorineural structures in the cochlea because of its TAT peptide carrier and good permeation through the round window membrane. It also possessed long pharmacologic activity due to a highly protease-resistant D-retro-inverso form (11). AM-111 has been shown to be effective in protecting hearing in the presence of various cochlear insults, such as noise trauma (11–15), acute labyrinthitis (16), cochlear implant electrode insertion trauma (17–19), aminoglycoside ototoxicity (11,18,20), and semicircular canal injury in otitis media (21). However, its effects on ischemic cochlear damage remain unclear. The purpose of the present study was to determine whether and how AM-111 can prevent ischemic damage of the cochlea by applying it on the round window.

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MATERIALS AND METHODS

Under approval by the ethics committee of Ehime University Graduate School of Medicine, the present study was conducted according to our institute's Guidelines for Animal Experimentation. The animals were housed in an animal room with a temperature of 21°C to 23°C and a 12/12-hour light/dark cycle (lights on: 7 AM to 7 PM). The animals had free access to food and water until the end of the experiment. All efforts were made to minimize the number of animals and suffering after the experimental protocol.

Adult 12- to 16-week-old Mongolian gerbils weighing 60 to 80 g were used. Transient cochlear ischemia was induced using a procedure described by Hata et al. (22). In Mongolian gerbils, the posterior communicating arteries of Willis' circle close spontaneously at 2 to 3 weeks after birth. Because in adults, the cochleae receive their blood supply solely from the vertebral arteries, cochlear ischemia is easily introduced by obstructing the bilateral vertebral arteries at the neck.

Anesthesia was introduced with a mixture of 3% halothane and nitrous oxide/oxygen (7:3) gas and then maintained with a mixture of 1% halothane gas. The animals were artificially ventilated using a ventilation tube inserted through the mouth. The tidal volume was set to 1 ml, and the rate was set to 70 times per minute. Body temperature was maintained at 37°C to 38°C with a heat lamp during the surgical procedure. The vertebral arteries were exposed bilaterally and dissected free from the surrounding connective tissue through a ventral transverse incision of the neck. Silk threads (4-0) were loosely looped around each artery, and the ischemia was induced on bilateral cochleae by pulling the ligatures with weights of 5 g for 15 minutes. The threads were subsequently removed to allow reperfusion, which was confirmed by observation with an operating microscope (Fig. 1). After these procedures, the otic bulla was opened to expose the round window. Next, 10 μl of AM-111 at a concentration of 1, 10, or 100 μM in a hyaluronic acid gel formulation (n = 6 for each group) was placed onto the round window membrane 30 minutes after ischemia. The gel formulation with no active substance (phosphate-buffered saline [PBS]) was used in the control group (n = 6). Finally, the wound was closed, and the animal was returned to the animal center. Separate from these studies, the effects of AM-111 in gel formulation placed onto the round window were investigated in 6 animals without induction of cochlear ischemia. This was performed to elucidate possible side effects of AM-111 by applying 100 μM of the agent. The experimental protocol was identical to that for ischemic animals.

For evaluation of auditory function, auditory brainstem responses (ABRs) were recorded using a signal processor (NEC Synax 1200, Tokyo, Japan) before and 4 and 7 days after the ischemic insult. Needle recording electrodes were placed in the vertex (reference), ipsilateral retroauricle (indifferent), and contralateral retroauricle (ground). Stimulus sounds were tone bursts of 8 kHz (0.5 ms rise/fall time), 4 kHz (1 ms rise/fall time), and 2 kHz (1 ms rise/fall time) with a plateau of 10 ms and stimulus rate of 9.5 Hz. They were applied in 10-dB steps, with 5-dB steps near the threshold. The responses were processed through a 50- to 3,000-Hz bandpass filter and averaged 300 times. Sound pressure in front of the tympanic membrane was monitored using a small microphone incorporated in a sound conduction tube. Sequential changes in hearing were assessed by comparing the thresholds of ABR before and after the insult.

Animals were sacrificed for histologic study on Day 7 after recordings of ABR. Under deep anesthesia, the animal was decapitated, and the otic bullae were removed. Each cochlea was intrascalarly perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 and postfixed for 2 hours with the same fixative at 4°C. It was then immersed in PBS. After removal of the lateral bony wall using sharp tweezers and microscissors, the organ of Corti at the basal turn was dissected out by means of surface preparation. According to our previous study, the basal turn is most vulnerable to ischemic insult (2) and was thus the preferred site for assessment of the protective effects of the agent. The specimens were stained for 30 minutes at room temperature with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) diluted 250 times in PBS containing 0.25% Triton C-100 and 1% bovine serum albumin. After rinsing in PBS, they were again stained with Hoechst 33342 (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA), dissolved in PBS, in a dark room for 1 hour. Specimens were then rinsed in PBS and mounted in carbonate-buffered glycerol (1 part 0.5 M carbonate buffer at pH 9.5 to 9 parts glycerol) containing 2.5% 1, 4 diazabicyclo [2,2,2] octane to retard bleaching of the fluorescent signal. Fluorescence was detected using an Olympus BX60 microscope equipped with green (BP 546, FT 580, LP 590 nm) and UV (BP 365, FT 395, LP 397 nm) filters. Intact and dead hair cells were

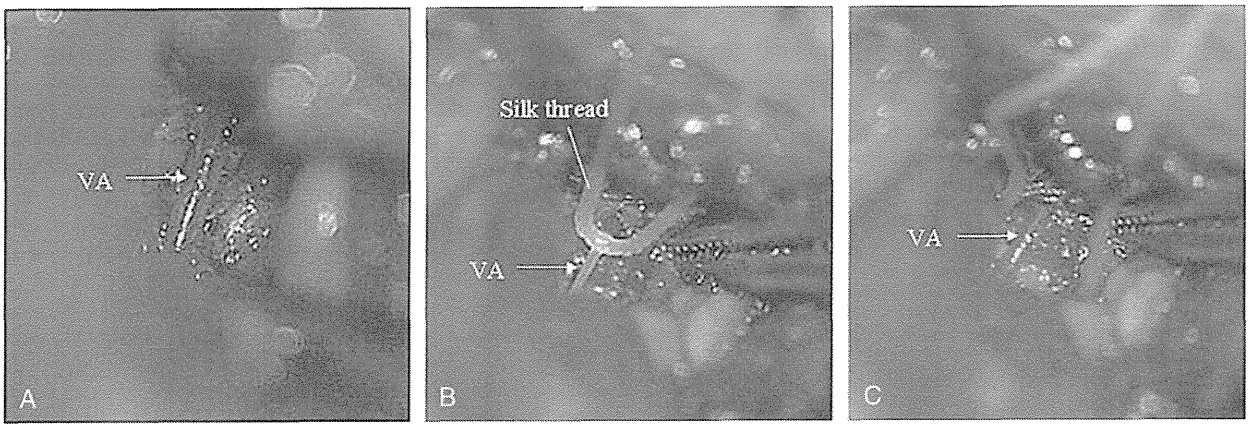

FIG. 1. Transient interruption of the cochlear blood flow. *A*, Exposure of the vertebral artery. *B*, Interruption of the cochlear blood flow by pulling silk thread looped around the artery. *C*, After loading transient ischemia, the thread was released and removed to allow recirculation.

counted, and the number of dead IHCs was calculated as a percentage of the whole number of IHCs.

The data were presented as means ± standard deviation. Statistical significance was assessed with a 1-way analysis of variance followed by Fisher's post hoc test. Values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS

Transient cochlear ischemia caused hearing loss immediately after the insult, which subsequently recovered to some extent over time. Figure 2 summarizes the increases in ABR thresholds at 2, 4, and 8 kHz on Days 4 and 7, with the threshold before ischemia being defined as 0 dB. In control animals (gel formulation with PBS), the ABR threshold increase at 8 kHz was 30.8 ± 5.3 dB on Day 4, which recovered slightly on Day 7 (25.0 ± 5.0 dB). The increase was mild at other frequencies: 4.2 ± 3.8 dB at 4 kHz and 5.0 ± 4.1 dB at 2 kHz on Day 7. Ischemic cochlear damage was attenuated by administration of AM-111. In animals treated with AM-111 at 100 μM, the ABR threshold increase at 8 kHz was 6.7 ± 2.4 dB on Day 4, which further improved to 3.3 ± 2.4 dB on Day 7. When animals were treated with AM-111 at 10 or 1 μM, the increase on Day 7 was 12.5 ± 2.5 dB and 16.7 ± 3.7 dB, respectively. Statistical analysis revealed that hearing impairment at 8 kHz was prevented by AM-111 at all 3 concentrations ($p < 0.01$ at 100 and 10 μM, and $p < 0.05$ at 1 μM). Hearing impairment at 4 and 2 kHz was rather mild in this animal model, and the effects of AM-111 were not evaluated at these frequencies.

Ischemic insult causes apoptotic cell death in the organ of Corti, and the damage is more severe in the IHCs than in the OHCs (5). In controls, the percentage of IHC loss at the basal turn was 13.3 ± 2.7% on Day 7, whereas that of OHC loss was 2.4 ± 0.7%. Figure 3 summarizes the loss of IHCs at 3 turns of the cochlea. As expected, the basal turn was most affected. Administration of AM-111 at 100 μM in gel formulation was proven effective in preventing ischemic damage to the cochlea: the mean IHC loss at the basal turn was only 3.1 ± 0.6%. At lower concentrations, the protective effects of AM-111 were less pronounced: IHC loss was 5.5 ± 0.7% in animals treated with the 10-μM concentration and 9.9 ± 1.2% in those treated with AM-111 at 1 μM. The effects were statistically significant ($p < 0.01$ at 100 and 10 μM, and $p < 0.05$ at 1 μM). The effects of AM-111 on OHC loss were unclear, as the OHC damage was minor in this animal model: the percentage of OHC loss at the basal turn was 1.5 ± 0.9%, 2.6 ± 0.8%, and 3.4 ± 1.1%, when treated with 100, 10 and 1 μM of the agent, respectively. Microscopic findings of the organ of Corti at the basal turn are shown in Figure 4.

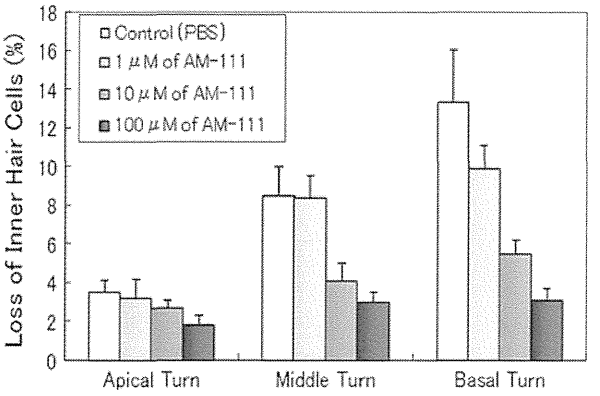

FIG. 3. Percentages of IHC loss at 3 turns of the cochlea and the effects of AM-111 in gel formulation placed on the round window. Administration of AM-111 prevented loss of IHCs at the basal turn (n = 6 for each group).

In animals without induction of cochlear ischemia, the ABR threshold increase on Day 7 after application of AM-111 at 100 μM was 0.8 ± 2.5 dB at 2, 4, and 8 kHz, respectively. These changes were minor and statistically not significant. Histologic study results also revealed that the inner ear was not affected by application of the agent.

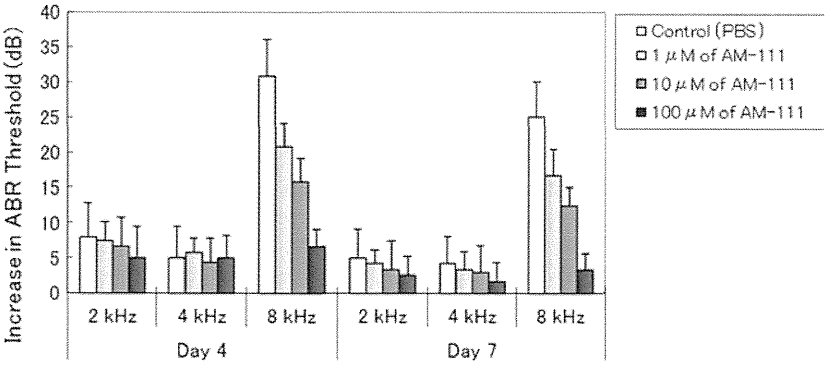

FIG. 2. ABR threshold increase after transient cochlear ischemia and the effects of AM-111 in gel formulation placed on the round window. ABR threshold before occlusion of the bilateral vertebral arteries was defined as 0 dB. Relative increases in ABR threshold at 2, 4, and 8 kHz and 1 standard deviation (n = 6 for each group) are represented. Administration of AM-111 prevented an increase in the ABR threshold, especially at 8 kHz, where it was most affected by ischemic insult. Effects of AM-111 were concentration dependent: AM-111 at 100 μM was more effective than that at 1 or 10 μM.

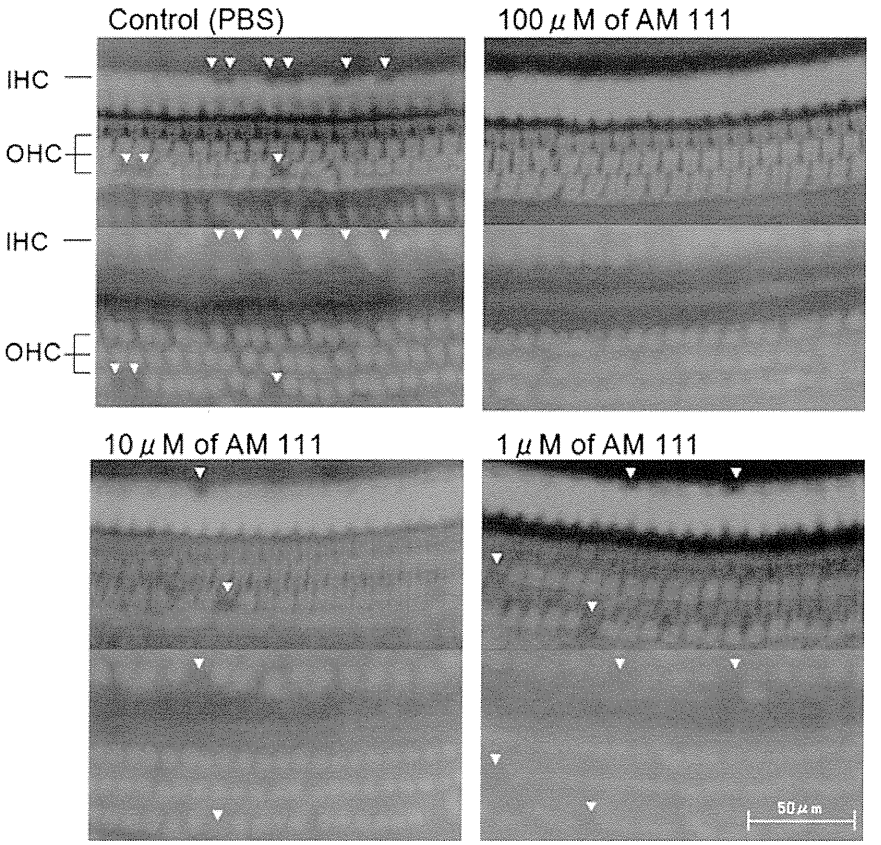

FIG. 4. Histologic findings of the cochlea at the basal turn on Day 7. *Triangles* indicate sites of hair cell loss. Rhodamine-phalloidin was used for staining cellular structures in red color and Hoechst 33342 for staining nuclei in blue color.

These findings suggest that AM-111 in gel formulation can be safely applied onto the round window without causing cochlear damage.

DISCUSSION

According to recent experimental studies, the JNK signaling pathway plays an important role in a cascade of programmed cell death (23). In the cochlea, various insults, such as loss of trophic factor (19), administration of ototoxic agents (11,18,20), and exposure to loud noise (11–15), were shown to cause apoptotic cell death of auditory neurons and hair cells through activation of the JNK pathway. AM-111, or D-JNKI-1 as it was previously named, is a novel medicine developed by Bonny et al. (24) to protect the cochlea by blocking the JNK pathway. It is now distributed for clinical and experimental studies by Auris Medical (Switzerland). The compound was effective in animal experiments in preventing neomycin ototoxicity and hearing loss due to acoustic trauma (14,15), semicircular canal injury (21), and acute labyrinthitis (16). In a study of focal cerebral ischemia, the active substance also was effective in the reduction of early calpain activation, late caspase 3 activation, and autophagosome formation, indicating involvement of the JNK pathway in 3 different types of cell death: necrosis, apoptosis, and autophagic cell death. Based on these findings, Ginet et al. (25) predicted that blocking the JNK pathway would be a novel modality in the treatment of brain ischemia in the future. In an experimental study in rats, it was further shown to be effective in preventing ischemic damage of the brain, even when administered 6 or 12 hours after the insult (26–28). The JNK inhibitor not only reduced the size of ischemic brain lesions but also lessened brain edema. Similar findings were noted in an animal model of cardiac ischemia (29).

In the present study, administration of AM-111 prevented an increase in the ABR threshold and loss of IHCs due to transient cochlear ischemia. The percentage of IHC loss caused by ischemia seemed rather great for the increase in ABR threshold. This is probably related to the cochlear anatomy, in which the IHCs are innervated much more densely than the OHCs. A single IHC is innervated by numerous nerve fibers, whereas a single nerve fiber innervates many OHCs. Correspondingly, the ABR threshold depends on whether the main lesion involves the OHC or IHC, although the hair cell damage is usually mixed. As previously stated, apoptosis is the dominant mode of cell death in this animal model (5). Various

mechanisms are supposedly involved in the process of cochlear cell death, such as excess production of free radicals, glutamate excitotoxicity, and failure of energy supply. Excess production of free radicals occurs as a process of ischemia/reperfusion injury that induces disintegration of the cell membrane through lipid peroxidation. Ischemic insult also facilitates expression of inducible nitric oxide (NO) synthase, a potent NO-synthesizing enzyme (30). Although NO is a reactive oxygen species and plays an important role in regulating vasoconstriction and neurotransmission, an excessive amount of NO leads to reactions with other molecules and increases the production of harmful free radicals, such as peroxynitrite radicals, which, in turn, cause hair cell loss by activation of JNK (14). Glutamate is a neurotransmitter released at the synapse between IHCs and primary auditory neurons. Ischemia leads to a drop in energy supply, increased depolarization, and excessive release of glutamate from the synaptic cleft, which then spreads to the surroundings (1,31). IHCs undergo apoptotic cell death as glutamate activates the JNK signaling pathway (26). This may explain why IHCs are more vulnerable to ischemic insult than OHCs, as shown by Amarjargal (32). Energy failure also causes cell death by a necrotic process. The cochlear sensory organ is sensitive to ischemic insult because in the cochlea, there is no storage of energy sources, such as glycogen. Neural structures in the inner ear use ATP as an energy source, which is produced during aerobic glycolysis. Because the glucose concentration is limited in the cochlea, existing ATP is soon exhausted by ischemia.

AM-111 is a water-soluble compound that can be intravenously administered. This is usually convenient and advantageous; however, delivery of a sufficient concentration of medicine into the cochlea is not always possible because the inner ear is protected by the blood-labyrinth barrier. Furthermore, systemic administration might cause unpredictable side effects. Alternatively, drug delivery through the round window has been proposed as an efficient way to supply pharmaceutical compounds into the cochlea. Wang et al. (13) used an osmotic mini-pump to administer AM-111 through the round window. Histologic examination showed that cellular uptake started as early as 30 minutes after AM-111 application. They concluded that the round window membrane was permeable not only to small molecules but, because of the intracellular transporter D-TAT, also to larger molecules such as the 31-amino-acid AM-111 peptide, with a molecular weight of 3820 Da. The authors also applied AM-111 in gel formulation directly on the round window. Using this procedure, the protective effect of AM-111 can be obtained with a single administration and a relatively low concentration of 100 μM.

The clinical development of AM-111 was initiated in 2006 with a prospective randomized phase I/II study in Germany by Suckfuell et al. (15). In the trial, a single dose of AM-111 at 0.4 or 2.0 mg/ml in gel formulation was administered by intratympanic injection to 11 patients with acute acoustic trauma within 24 hours after noise exposure. The results were encouraging: the pure-tone average at 4 and 6 kHz before treatment was 36 ± 16 dB, and this improved by 11 ± 12 dB after 3 days and 11 ± 14 dB after 30 days of treatment with AM-111. Currently, a much larger phase IIb clinical trial is ongoing in several European countries to treat patients with acute noise trauma or ISSNHL within 48 hours from onset. Cochlear ischemia is one of several possible origins of ISSNHL.

In conclusion, AM-111, an inhibitor of the JNK signaling pathway, can prevent ischemic damage of the cochlea when administered shortly after the incident. Application of the medicine on the round window in a gel formulation could be a promising procedure in the treatment of acute sensorineural hearing loss triggered not only by loud noise, surgical trauma, and ototoxic agents but also by cochlear ischemia.

Acknowledgment: AM-111 was supplied courtesy of Dr. Thomas Meyer of Auris Medical.

REFERENCES

1. Hakuba N, Gyo K, Yanagihara N, Mitani A, Kataoka K. Efflux of glutamate into the perilymph of the cochlea following transient ischemia in the gerbil. *Neurosci Lett* 1997;230:69–71.
2. Koga K, Hakuba N, Watanabe F, Shudou M, Nakagawa T, Gyo K. Transient cochlear ischemia causes delayed cell death in the organ of Corti: an experimental study in gerbils. *J Comp Neurol* 2003;456:105–11.
3. Watanabe F, Hakuba N, Gyo K. Measurement of DPOAE after ischemia/reperfusion injury of the cochlea in gerbils. *Neurosci Lett* 2009;467:135–8.
4. Hakuba N, Koga K, Shudou M, Watanabe F, Mitani A, Gyo K. Hearing loss and glutamate efflux in the perilymph following transient hindbrain ischemia in gerbils. *J Comp Neurol* 2000;418:217–26.
5. Taniguchi M, Hakuba N, Koga K, Watanabe F, Hyodo J, Gyo K. Apoptotic hair cell death after transient cochlear ischemia in gerbils. *Neuroreport* 2002;13:2459–62.
6. Hakuba N, Matsubara A, Hyodo J, et al. AMPA/kainate-type glutamate receptor antagonist reduces progressive inner hair cell loss after transient cochlear ischemia. *Brain Res* 2003;979:194–202.
7. Maetani T, Hakuba N, Taniguchi M, Hyodo J, Shimizu Y, Gyo K. Free radical scavenger protects against inner hair cell loss after cochlear ischemia. *Neuroreport* 2003;14:1881–4.
8. Fujita K, Hakuba N, Hata R, et al. Ginsenoside Rb1 protects against damage to the spiral ganglion cells after cochlear ischemia. *Neurosci Lett* 2007;415:113–7.
9. Fujiwara T, Hato N, Nakagawa T, et al. Insulin-like growth factor 1 treatment via hydrogels rescue cochlear hair cells from ischemic injury. *Neuroreport* 2008;19:1585–8.
10. Maetani T, Hyodo J, Takeda S, Hakuba N, Gyo K. Prednisolone prevents transient ischemia-induced cochlear damage in gerbils. *Acta Otolaryngol* 2009;562:24–27.
11. Wang J, Van De Water TR, Bonny C, de Ribaupierre F, Puel JL, Zine A. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. *J Neurosci* 2003;23:8596–7.
12. Pirvola U, Xing-Qun L, Virkkala J, et al. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. *J Neurosci* 2000;20:43–50.
13. Wang J, Ruel J, Ladrech S, Bonny C, van de Water TR, Puel JL. Inhibition of the c-Jun N-terminal kinase-mediated mitochondrial cell death pathway restores auditory function in sound-exposed animals. *Mol Pharmacol* 2007;71:654–66.
14. Coleman JKM, Littlesunday C, Jackson R, Meyer T. AM-111

protects against permanent hearing loss from impulse noise trauma. *Hear Res* 2007;226:70–8.
15. Suckfuell M, Canisi S, Striethi S, Scherer H, Haisch A. Intratympanic treatment of acute acoustic trauma with a cell-permeable JNK ligand: a prospective randomized phase I/II study. *Acta Otolaryngol* 2007; 127:938–42.
16. Barkdull GC, Hondarrague Y, Meyer T, Harris JP, Keithley EM. AM-111 reduces hearing loss in a guinea pig model of acute labyrinthitis. *Laryngoscope* 2007;117:2174–82.
17. Eshraghi AA, He J, Mou CH, et al. D-JNKI-1 treatment prevents the progression of hearing loss in a model of cochlear implantation trauma. *Otol Neurotol* 2006;27:504–11.
18. Eshraghi AA, Wang J, Adil E, et al. Blocking c-Jun-N-terminal kinase signaling can prevent hearing loss induced by both electrode insertion trauma and neomycin ototoxicity. *Hear Res* 2007;226: 168–77.
19. Scarpidis U, Madnani D, Shoemaker C, et al. Arrest of apoptosis in auditory neurons: implications for sensorineural preservation in cochlear implantation. *Otol Neurotol* 2003;24:409–17.
20. Ylikoski J, Xing-Qun L, Virkkala J, Pirvola U. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. *Hear Res* 2002;166:33–43.
21. Grindal TC, Sampson EM, Antonelli PJ. AM-111 prevents hearing loss from semicircular canal injury in otitis media. *Laryngoscope* 2010;120:178–82.
22. Hata R, Matsumoto M, Kitagawa K, et al. A new gerbil model of hindbrain ischemia by extracranial occlusion of the lateral ertebral arteries. *J Neurol Sci* 1994;121:79–89.
23. Tournier C, Hess P, Yang DD, et al. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 2000;288:870–4.
24. Bonny C, Oberson A, Negri S, et al. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes* 2001; 50:77–82.
25. Ginet V, Puyal J, Magnin G, Clarke PG, Truttmann AC. Limited role of the c-Jun N-terminal kinase pathway in a neonatal rat model of cerebral hypoxia-ischemia. *J Neurochem* 2009;108:552–62.
26. Liu JR, Zhao Y, Patzer A, et al. The JNK-inhibitor XG-102 enhances the neuroprotection of hyperbaric oxygen after cerebral ischaemia in adult rats. *Neuropathol Appl Neurobiol* 2010;36: 211–24.
27. Repici M, Centeno C, Tomasi S, et al. Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation. *Neuroscience* 2007;150: 40–9.
28. Borsello T, Clarke PG, Hirt L, et al. A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med* 2003;9:1180–6.
29. Milano G, Morel S, Bonny C, et al. A peptide inhibitor of c-Jun NH2-terminal kinase reduces myocardial ischemia-reperfusion injury and infarct size in vivo. *Am J Physiol Heart Circ Physiol* 2007;292:1828–35.
30. Morizane I, Hakuba N, Hyodo J, et al. Ischemic damage increases nitric oxide production via inducible nitric oxide synthase in the cochlea. *Neurosci Lett* 2005;391:62–7.
31. Hyodo J Hakuba N, Hato N, et al. Glutamate agonist causes irreversible degeneration of inner hair cells. *Neuroreport* 2009;20: 1255–9.
32. Amarjargal N, Andreeva N, Gross J, et al. Differential vulnerability of outer and inner hair cells during and after oxygen-glucose deprivation in organotypic cultures of newborn rats. *Physiol Res* 2009;58:895–902.

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Liposome-Encapsulated Hemoglobin Alleviates Hearing Loss After Transient Cochlear Ischemia and Reperfusion in the Gerbil

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Abstract: To test liposome-encapsulated hemoglobin (LEH) in transient cochlear ischemia/reperfusion as a model of sudden deafness, Mongolian gerbils were randomly assigned to receive 2 mL/kg of either low-affinity LEH (l-LEH, $P_{50}O_2 = 40$ mm Hg), high-affinity LEH (h-LEH, $P_{50}O_2 = 10$ mm Hg), homologous red blood cells (RBCs), or saline (each group $n = 6$) 30 min before 15-min occlusion of the bilateral vertebral arteries and reperfusion. Sequential changes in hearing were assessed by auditory brain response 1, 4, and 7 days after ischemia/reperfusion, when the animals were sacrificed for pathological studies. h-LEH was significantly more protective than l-LEH in suppressing hearing loss, in contrast to RBC or saline treatment, at 8, 16, and 32 kHz, where hearing loss was most severe ($P < 0.05$ between any two groups) on the first day after cochlear ischemia/reperfusion. Thereafter, hearing loss improved gradually in all groups, with a significant difference among groups up to 7 days, when morphological studies revealed that the inner hair cells but not the outer hair cells, were significantly lost in the groups in the same order. The results suggest that pretreatment with h-LEH is significantly more protective than l-LEH in mitigating hearing loss and underlying pathological damage, in contrast to transfusion or saline infusion 7 days after transient cochlear ischemia/reperfusion. **Key Words:** Artificial oxygen carrier—Auditory brain response—Cochlear ischemia—Sudden deafness—Reperfusion injury.

Acute interruption of blood supply is considered to be the primary cause of sudden deafness (1), a rare but serious otological event that affects 20 people per 100 000 per year (2). Effective treatments for this condition have, however, been quite limited (3,4), and approaches for improving the demand/supply balance of oxygen (O_2) by local hypothermia (5,6) to reduce demand or hyperbaric O_2 therapy to increase supply remain controversial. Thus, we tested the effects of liposome-encapsulated hemoglobin (LEH) (7,8), the efficacy of which has been reported in reducing focal ischemic injury of rat brain (9–11) as well as of nonhuman primates (12) as an artificial O_2 carrier, but not as a substitute of red blood cells (RBCs) for transfusion (13,14). The liposome capsule is small enough (230 nm) to circulate with plasma through capillaries and collaterals, thereby shortening O_2 diffusion distance in ischemic tissues. At the same time, LEH is large enough to remain in the vascular lumen, extending its retention time and avoiding direct contact of hemoglobin with the vascular endothelium (7,8). As the Mongolian gerbil lacks the posterior communicating arteries of the circle of Willis, occlusion of the bilateral vertebral artery causes hindbrain ischemia; this model has been used as an animal model of transient cochlear ischemia (5,6,15,16). In this study, we examined the effects of LEH with high- and low-O_2 affinity on ischemia and reperfusion injury to the cochlea in terms of hearing loss as determined by auditory brain

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response (ABR) and the severity of damage to the inner and outer hair cells (OHCs) in the gerbil as a simulation of clinical sudden deafness.

MATERIALS AND METHODS

The experiments were conducted in accordance with the Guidelines for Animal Experimentation at Ehime University Graduate School of Medicine. The animals received humane care as required by the institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* (17).

LEH

The relevant characteristics of LEH (Terumo, Tokyo, Japan) have been reported elsewhere (7). Briefly, the liposome capsule, 230 nm in mean diameter, contains purified hemoglobin from outdated human RBCs. The liposome capsule is coated with polyethylene glycol to reduce mutual aggregation, to avoid recognition by the reticuloendothelial cell system, and to prolong its half-life in the circulation (8). In its preparation, inositol hexaphosphate was used to control O_2 affinity to $P_{50}O_2 = 40$ mm Hg for low-affinity LEH (l-LEH) and to $P_{50}O_2 = 10$ mm Hg for high-affinity LEH (h-LEH) (Fig. 1). LEH, suspended in saline at a hemoglobin concentration of 6 g/dL or 20% by volume, has reduced viscosity (2 cP) compared to blood (5 cP), and specific gravity close to that of plasma. Homologous RBCs were washed with saline three times and suspended in saline at a final concentration of 20% hematocrit.

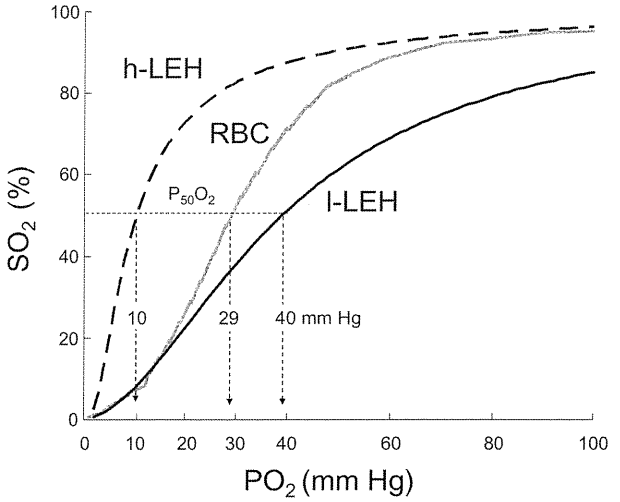

FIG. 1. Oxygen dissociation characteristics for h-LEH, l-LEH, and gerbil's RBC. Oxygen dissociation characteristics have been determined for every batch of LEH by actually measuring O_2 saturation using a UV-Spectrophotometer (MPS-2000, Shimadzu, Kyoto, Japan) in closed cells under a varying range of O_2 (0 to 95%) and 5% CO_2 with N_2 replacement. Iron oxidation state was determined by cyanmethemoglobin method. The equilibrium curve for RBC was from gerbil's RBC ($P_{50}O_2 = 28.7$ mm Hg). While l-LEH ($P_{50}O_2 = 40$ mm Hg) is considered to have higher O_2 delivery than gerbil's RBC ($P_{50}O_2 = 28.7$ mm Hg) under physiologic conditions, h-LEH ($P_{50}O_2 = 10$ mm Hg) is considered to have more efficient O_2 delivery than gerbil's RBC under hypoxic condition.

Experimental animals

Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 60–80 g were purchased from Kyudo (Tosu, Japan) and used at 12–16 weeks of age. Anesthesia was induced with a mixture of 3% halothane and nitrous oxide:oxygen (7:3) gas, and was maintained with a mixture of 1% halothane gas. The animals were ventilated artificially via a transoral tracheal tube (tidal volume 1 mL; respiration rate 70/min). During the experiment, body temperature was monitored with a thermocouple probe (PTI-200, Unique Medical, Tokyo, Japan) placed in the rectum and kept at 37 ± 1°C using a heating pad (HP-1 M, Physitemp, Clifton, NJ, USA). The femoral vein was exposed to establish an intravenous infusion line by polyethylene catheter. The animals were randomly assigned to receive 2 mL/kg of h-LEH ($n = 6$), l-LEH ($n = 6$), RBCs ($n = 6$), or saline ($n = 6$) 30 min before cochlear ischemia over 10 min to avoid acute volume load; after which, the catheter was removed and the vein was ligated.

Transient cochlear ischemia and reperfusion

Thirty minutes after administration of the solutions, cochlear ischemia and reperfusion were effected as previously described (15). Briefly, with the animal in supine position, the vertebral arteries were exposed bilaterally (Fig. 2A) and dissected free from the surrounding tissues through a ventral midline incision in the neck. Silk ligatures (4-0) were looped loosely around each artery. Ischemia was then induced in both cochleae by pulling on the ligatures (Fig. 2B) simultaneously using 5-g weights for 15 min. Subsequently, the threads were removed to allow reperfusion, which was confirmed by observation through an operating microscope. The wound was closed and the animals were returned to cages with water and food ad libitum until further testing. As a preliminary study, changes in cochlear blood flow during the bilateral vertebral artery occlusion were monitored by laser Doppler flow meter (ALF-21, Advance, Tokyo, Japan) focused on the lateral aspect of its basal turn in six animals. The percent level of cochlear blood flow was calculated, with the value measured after exsanguination taken as 0% and the preischemic value regarded as 100%.

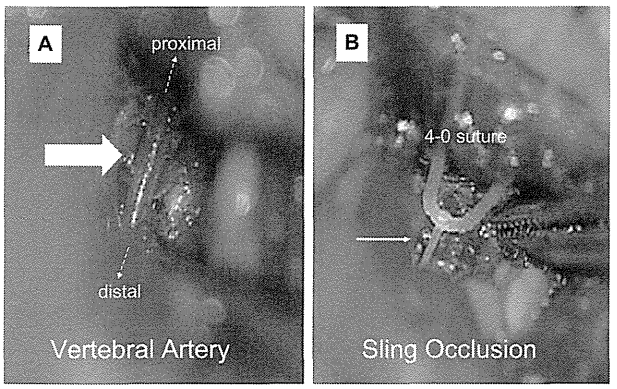

FIG. 2. Intact vertebral artery (A) and occlusion by pulling the sling (B). Silk ligatures (4-0) were looped loosely around bilateral vertebral artery. Ischemia was induced in both cochleae by pulling the ligatures simultaneously using 5-g weights for 15 min. Releasing and removing these slings allowed reperfusion of the cochlear artery.

Evaluation of hearing by ABR

The hearing of each animal was assessed before and at 1, 4, and 7 days after ischemia and/or reperfusion. The animal was anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (1 mg/kg), and then ABR was recorded using a signal processor (NEC Synax 1200, NEC Medical Systems, Tokyo, Japan). Recording and reference needle electrodes were placed at the vertex and retroauricular area, respectively. The stimulus sound was a pure tone burst at 8, 16, and 32 kHz (rise and fall time, 1 ms; duration, 5 ms; repetition rate, 15/s) delivered in an open field system (DPS-725, DIA Medical, Tokyo, Japan). The phases of the stimuli were alternated, thereby negating interference of the cochlear microphonics. The speaker was located a constant 20 cm from the left external auditory canal. Responses to 1000 consecutive stimuli were averaged. The ABR threshold was determined by recording responses in 5-dB steps.

Histological study

For the histological study, the animals were decapitated under deep anesthesia 7 days after ischemia. After removing the otic bullae, the cochleae were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 into the scala tympani and postfixed for 2 h with the same fixative at 4°C. The specimens were immersed in phosphate-buffered saline (PBS) and the organ of Corti was dissected using a surface preparation technique under an operating microscope. The walls of the bony cochleae were removed entirely without disrupting the organ of Corti. Then, the basal turn of the organ of Corti was isolated. The specimen was stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) diluted 250 times in PBS containing 0.25% Triton X-100 and 1% bovine serum albumin for 30 min at room temperature. After rinsing in PBS, it was further stained with Hoechst 33342 (Calbiochem-Novabiochem, La Jolla, CA, USA) dissolved in PBS in a dark room for 1 h. It was again rinsed in PBS and mounted in carbonate-buffered glycerol (one part 0.5 M carbonate buffer at pH 9.5 to nine parts glycerol) containing 2.5% 1,4-diazabicyclo[2,2,2]octane to retard bleaching of the fluorescent signal. Fluorescence was detected using an Olympus BX60 microscope (Olympus, Tokyo, Japan) equipped with green (band pass filter [BP] 546, Farb Teiler Spiegel [FT] 580, long pass filter [LP] 590 nm) and UV (BP 365, FT 395, LP 397 nm) filters. Rhodamine-phalloidin staining permits observation of the hair cell stereocilia, whereas Hoechst 33342 staining reveals that of the nuclei. The numbers of intact and dead hair cells at the basal turn were counted, and the percentage of dead hair cells to intact hair cells (IHCs) was determined. As gerbils have about 300 IHCs at the basal turn, we examined at least 200 IHCs in each specimen.

Statistics

All data are presented as mean ± SD. Statistical differences between groups were evaluated using the Mann–Whitney *U*-test. The results were considered significant at $P < 0.05$.

RESULTS

Cochlear ischemia

Occlusion of the bilateral vertebral arteries (Fig. 2) severely reduced cochlear blood flow as measured by laser Doppler flow meter (Fig. 3), which then increased over the preischemic level and returned to baseline shortly after reperfusion.

Hearing loss

The preischemic ABR threshold was set at 0 dB, and the subsequent increase (hearing impairment) in threshold is shown on the ordinate (Fig. 4). At 8 kHz (left panel), the increase in ABR threshold was significantly suppressed in the order of the animals treated with h-LEH, l-LEH, and RBCs or saline. Among the frequencies tested (8, 16, and 32 kHz), the higher the frequency, the more severe the magnitude of hearing loss and difference among the treatment groups. The magnitude of hearing loss was most prominent on day 1, then declining in severity with time to day 7 at each frequency tested and in every

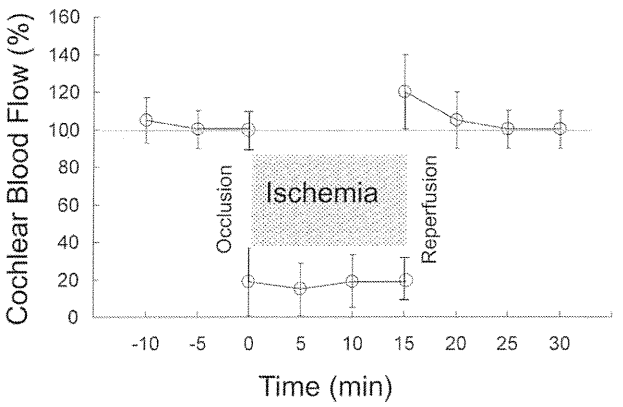

FIG. 3. Occlusion of the bilateral vertebral arteries severely reduced cochlear blood flow before reperfusion, which then increased over the preischemic level after reperfusion, and returned to baseline shortly thereafter.

treatment group. This means that hearing loss remained more severe in the order of animals treated with RBC or saline, l-LEH, and H-LEH at 32 kHz on day 7 (right). In contrast, hearing impairment severity was milder in each group with the h-LEH-treated animals recovering to the preischemic level at 8 kHz.

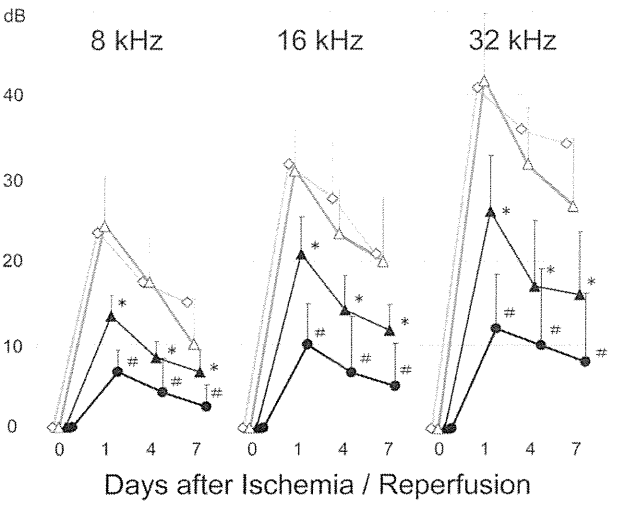

FIG. 4. The average increase in ABR threshold was significantly milder (all $P < 0.05$) in the order of animals receiving h-LEH (closed circles), l-LEH (closed triangles), and RBCs (open triangles) or saline (open rectangles). No difference was observed between the latter two groups. Among the frequencies tested, the higher the frequency, the more severe was the magnitude of hearing loss and the difference among the treatment groups. Hearing loss was most prominent on day 1, then decreasing in severity with time to day 7 in each frequency or treatment group, keeping the significant difference among animals treated with h-LEH, l-LEH, and animals receiving RBCs or saline; the hash symbol (#) and asterisk (*) indicate a significant difference ($P < 0.05$) from the other groups.

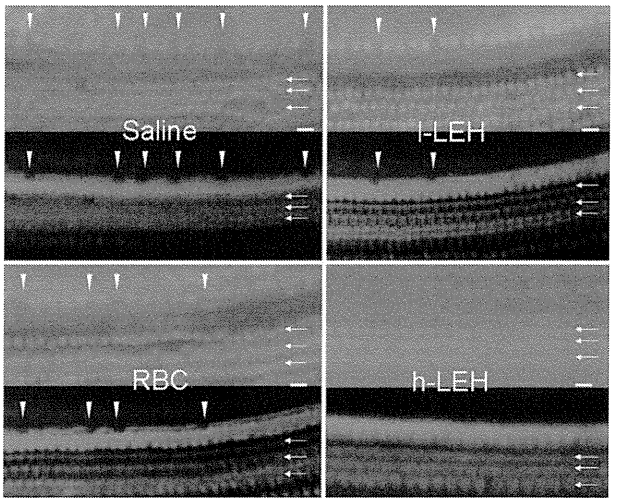

FIG. 5. While the stereocilia (rhodamine staining, red, bottom) and nuclei (Hoechst 33342, blue, top) of some IHCs had sporadically disappeared (vertical arrowheads) in the saline- and RBC-treated groups, such phenomena were less frequent in the LEH-treated groups. OHCs (horizontal arrows) remained essentially intact. Scale bar indicates 20 μm.

Morphological findings

Representative epifluorescence images of the organ of Corti from one animal in each group 7 days after ischemia (Fig. 5) showed that the stereocilia (rhodamine-phalloidin staining, red, bottom) and (Hoechst 33342 staining, blue, top) of the IHCs simultaneously disappeared, indicating cell loss. Such sporadic IHC loss contrasted sharply with the underlying OHCs, which remained mostly intact. The average ratio of dead/intact cells of IHCs on day 7 (Fig. 6) was significantly smaller in the animal groups pretreated with h-LEH (3.7 ± 1.2%, $P < 0.01$) or l-LEH (6.5 ± 1.2%, $P < 0.01$), and was less with RBCs (15.0 ± 2.2%) or saline (14.5 ± 2.5%). The latter two groups were almost equal, showing no significant difference. In contrast, cell loss was much less frequent in OHCs, with no significant difference among the treatment groups.

DISCUSSION

As acute interruption of the blood supply to the cochlea is considered to be one of the major causes of sudden deafness (1,2), remedies have been proposed to treat cochlear hypoxia. Nonetheless, widely accepted therapeutic approaches have been limited (3,4), suggesting the complex nature of the mechanism(s) involved and the variability of symptoms due in part to the differential vulnerability of the hindbrain to hypoxia (15). This led us, in the current study, to examine the effect of LEH as an artificial O_2

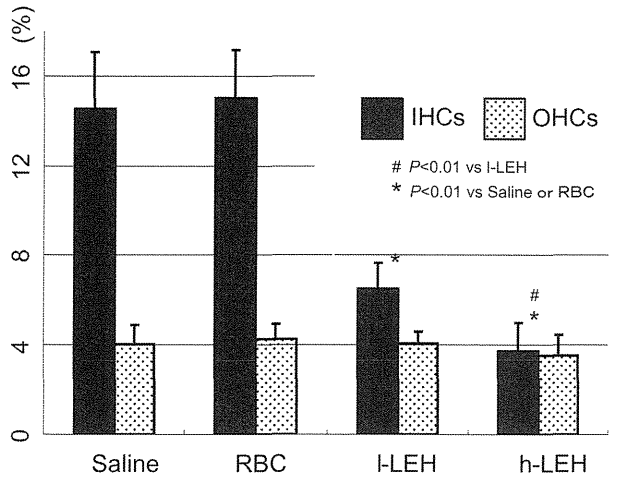

FIG. 6. Average cell loss at the basal turn 7 days after ischemia/reperfusion was more prominent in IHCs, significantly less frequent in the order of animals receiving h-LEH, l-LEH, and saline or RBCs, than in OHCs, which remained essentially intact. # $P < 0.01$ vs l-LEH; * $P < 0.01$ vs Saline or RBC.

carrier with the aim of increasing the O_2 supply, and it was revealed that the average increase in ABR threshold was significantly milder in the order of animals pretreated with h-LEH, l-LEH, and RBCs or saline. Morphologically, the disappearance of stereocilia and nuclei of IHCs in the organ of Corti was clearly correlated with the functional results, with significant differences among the treatment groups.

As the cochlea is solely perfused by the common cochlear artery as an end artery with few collaterals (18), there was severe reduction in cochlear blood flow during occlusion of the bilateral vertebral arteries (Fig. 3). Nonetheless, it remains unclear whether the blood flow was completely interrupted or persisted at least to some degree, as laser Doppler measurements reflect the average RBC flow in the area exposed to laser. In fact, plasma flow might have been different, as in the observation of Villringer et al. (19), who reported that plasma flow decreased along with RBC flow, but it still remained homogeneous and persistent even to the ischemic core. Therefore, it is plausible that LEH flows with plasma, shortens the O_2 diffusion distance, and thereby suppresses hearing defect (function) as well as IHC loss (morphology), leading to preservation of cochlear integrity. This finding is in agreement with our previous observation (16) that ABR threshold elevation caused by cochlear ischemia was due mainly to the damage to IHCs, which is considered more vulnerable to ischemia due to their high energy consumption, as compared with OHCs.

The current results disclosed that h-LEH was more effective than l-LEH in preventing ischemic and/or reperfusion injury to the inner ear. We consider that the high sensitivity and specificity of ABR allowed delineation of functional differences between treatment groups, in contrast to our previous studies that showed no apparent neurological improvements despite morphological differences (9,20). Quantitative morphometry, counting most of the IHCs in each animal, might also have allowed precise delineation of the morphological effects of each treatment. These results were in accordance with our recent observation that h-LEH provided a similar level of protection at a 1/5 to 1/25 dosage as that of l-LEH after middle cerebral artery (MCA) occlusion and reperfusion (11). As O_2 affinity is the only difference between these LEHs, the superiority of h-LEH over l-LEH may derive from the greater and more efficient O_2 delivery (Fig. 1) to tissues under hypoxic condition, or targeted O_2 delivery to ischemic cochlear tissue.

The global hindbrain ischemia induced in the current study may differ from the previous application of LEHs to focal brain ischemia (9–12), where LEH was reported to improve microcirculation to the periphery of the ischemic focus (21) or penumbral lesion, thereby eventually reducing the extent of infarction (22). Instead, the current model induced bilateral and global ischemia and reperfusion injury to the hindbrain, including the brain stem and cerebellum, which can result in respiratory arrest, convulsion, and death. For survival and long-term observation, the length of ischemia was limited to a maximum of 15 min, which was much shorter than in our previous experiments in MCA regions (9–12). This may have altered the severity of ischemia and reperfusion injury, cell survival, and apoptosis, and therefore the presentation of functional as well as morphological sequelae. Sporadic versus concentric distribution of neural cell loss may have been due to these factors. While the tendency toward functional recovery is common, the improvement was considered to be due to neural compensation rather than recovery of the affected neuronal tissues. As the development of edema may further reduce blood flow and aggravate neuronal cell loss according to the vulnerability to ischemia (15), LEH may be protective in such a global ischemia and reperfusion response (23), as it was observed in our previous study (10) that the reduction of brain edema by LEH was not limited just to the focus of ischemia in the MCA region but rather extended to the ipsilateral hippocampus and even to the pyriform lobe and contralateral hippocampus. We previously reported that

transient cochlear ischemia causes a remarkable increase in nitric oxide production in the perilymph, and that this is attributable to the inducible nitric oxide synthase (iNOS) pathway (24). As the iNOS gene includes the hypoxia-responsive element (25), oxygen delivery could alleviate reperfusion injury by decreasing iNOS gene activation. Therefore, it is possible that the antioxidant property may also be involved, may be more potent in h-LEH, and may be able to better protect ischemic tissues from reperfusion injury. Although the animals were pretreated in order to examine the maximum effects of LEHs in the current study, it is necessary to examine the efficacy of LEHs used therapeutically or even after reperfusion in order to delineate the effects on reperfusion.

CONCLUSION

The results showed that pretreatment with LEH could alleviate hearing impairment (function) as well as inner hair cell loss (morphology) for up to 7 days following transient cochlear ischemia and reperfusion. While the results suggest that more efficient O_2 delivery (l-LEH < h-LEH) during ischemia became apparent after reperfusion as in our previous experiments (9–12), the possible involvement of the antioxidant property of LEH cannot be ruled out. Therefore, timing, O_2 affinity, and dosage need to be explored during a longer-term observation to determine the effects of LEH on cochlear ischemia and reperfusion in an experimental model of sudden deafness.

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REFERENCES

1. Kim JS, Lopez I, DiPatre PL, Liu F, Ishiyama A, Balow RW. Internal auditory artery infarction: clinicopathologic correlation. *Neurology* 1999;52:40–4.
2. Fetterman BL, Saunders JE, Luxford WM. Prognosis and treatment of sudden sensorineural hearing loss. *Am J Otol* 1996;17:529–36.
3. Conlin AE, Parnes LS. Treatment of sudden sensorineural hearing loss: I. A systematic review. *Arch Otolaryngol Head Neck Surg* 2007;133:573–81.
4. Makara GB, Haller J. Non-genomic effects of glucocorticoids in neural system. Evidence, mechanisms and implications. *Prog Neurobiol* 2001;65:367–90.
5. Watanabe F, Koga K, Hakuba N, Gyo K. Hypothermia prevents hearing loss and progressive hair cell loss after transient cochlear ischemia in gerbils. *Neuroscience* 2001;102:639–45.
6. Takeda S, Hakuba N, Yoshida T, et al. Postischemic mild hypothermia alleviates hearing loss because of transient ischemia. *Neuroreport* 2008;19:1325–8.
7. Ogata Y. Characteristics and function of human hemoglobin vesicles as an oxygen carrier. *Polym Adv Technol* 2000;11: 205–9.
8. Kaneda S, Ishizuka T, Goto H, Kimura T, Inaba K, Kasukawa H. Liposome-encapsulated hemoglobin, TRM-645: current status of the development and important issues for clinical application. *Artif Organs* 2009;33:146–52.
9. Kawaguchi AT, Fukumoto D, Haida M, Ogata Y, Yamano M, Tsukada H. Liposome-encapsulated hemoglobin reduces the size of cerebral infarction in the rat: evaluation with photochemically induced thrombosis of the middle cerebral artery. *Stroke* 2007;38:1626–32.
10. Kawaguchi AT, Kurita D, Furuya H, Yamano M, Ogata Y, Haida M. Liposome-encapsulated hemoglobin alleviates brain edema after permanent occlusion of the middle cerebral artery in the rat. *Artif Organs* 2009;33:153–8.
11. Fukumoto D, Kawaguchi AT, Haida M, Yamano M, Ogata Y, Tsukada H. Liposome-encapsulated hemoglobin reduces the size of cerebral infarction in the rat: effect of oxygen affinity. *Artif Organs* 2009;33:159–63.
12. Kawaguchi AT, Haida M, Yamano M, Fukumoto D, Ogata Y, Tsukada H. Liposome-encapsulated hemoglobin ameliorates ischemic stroke in nonhuman primates: an acute study. *J Pharmacol Exp Ther* 2010;332:429–36.
13. Nogami Y, Kinoshita M, Takase B, et al. Liposome-encapsulated hemoglobin transfusion rescues rats undergoing progressive hemodilution from lethal organ hypoxia without scavenging nitric oxide. *Ann Surg* 2008;248:310–9.
14. Yoshiba F, Kawaguchi AT, Hyodo O, Kinoue T, Inokuchi S, Kato S. Possible role of artificial oxygen carrier in transfusion medicine: a retrospective analysis on current transfusion practice. *Artif Organs* 2009;33:127–32.
15. Hata R, Matsumoto M, Hatakeyama T, et al. Differential vulnerability in the hindbrain neurons and local cerebral blood flow during bilateral vertebral occlusion in gerbils. *Neuroscience* 1993;56:423–39.
16. Hakuba N, Koga K, Shudou M, Watanabe F, Mitani A, Gyo K. Hearing loss and glutamate efflux in the perilymph following transient hindbrain ischemia in gerbils. *J Comp Neurol* 2000;418:217–26.

17. Institute of Laboratory Animal Resources. *Guide for the Care and Use of Laboratory Animals*, 7th Edition. Washington, DC: Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996.
18. Nakashima T, Naganawa S, Sone M, et al. Disorders of cochlear blood flow. *Brain Res Rev* 2003;43:17–28.
19. Villringer A, Them A, Lindauer U, Einhaupl K, Dirnagl U. Capillary perfusion of the rat brain cortex. An in vivo confocal microscopy study. *Circ Res* 1994;75:55–62.
20. Kawaguchi AT, Nakai K, Fukumoto D, Yamano M, Haida H, Tsukada H. *S*-nitrosylated pegylated hemoglobin reduces the size of cerebral infarction in rats. *Artif Organs* 2009;33: 183–8.
21. Urakami T, Kawaguchi AT, Akai S, et al. In vivo distribution of liposome-encapsulated hemoglobin determined by positron emission tomography. *Artif Organs* 2009;33:164–8.
22. Dirnagl U, Iadecola C, Moskowitz MA. Pathology of ischemic stroke: an integrated view. *Trends Neurosci* 1999;22:391–7.
23. Taylor JM, Crack PJ. Impact of oxidative stress on neural survival. *Clin Exp Pharmacol Physiol* 2004;31:397–406.
24. Morizane I, Hakuba N, Hyodo J, et al. Ischemic damage increases nitric oxide production via inducible nitric oxide synthase in the cochlea. *Neurosci Lett* 2005;391:62–7.
25. Kainanen R, Vartiainen N, Koistinaho J. Molecular cloning and characterization of the rat inducible nitric oxide synthase (iNOS) gene. *Gene* 1999;234:297–305.

Ⅴ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表
発表業績

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○Okamoto Y, Mutai H, Nakano A, Arimoto Y, Sugiuchi T, Masuda S, Morimoto N, Sakamoto H, Ogahara N, Takagi A, Taiji H, Kaga K, Ogawa K, Matsunaga T. Subgroups of enlarged vestibular aqueduct in relation to SLC26A4 mutations and hearing loss. Laryngoscope. 2013 Sep 18. doi: 10.1002/lary.24368. [Epub ahead of print]

○Mizutari K, Michikawa T, Saito H, Okamoto Y, Enomoto C, Takebayashi T, Ogawa K, Nishiwaki Y. Age-related hearing loss and the factors determining continued usage of hearing aids among elderly community-dwelling residents. PLoS One 2013; 8(9): e73622.

○Sano H, Okamoto M, Ohhashi K, Ino T, Iwasaki S, Ogawa K. Self-reported symptoms in patients with idiopathic sudden sensorineural hearing loss. Otol Neurotol 2013; 34(8): 1405-10.

Takiguchi Y, Sun GW, Ogawa K, Matsunaga T. Long-lasting changes in the cochlear K+ recycling structures after acute energy failure. Neurosci Res 2013; 77(1-2): 33-41.

○Watabe T, Matsunaga T, Namba K, Mutai H, Inoue Y, Ogawa K. Moderate hearing loss associated with a novel KCNQ4 non-truncating mutation located near the N-terminus of the pore helix. Biochem Biophys Res Commun 2013; 432(3): 475-9.

○Kanzaki S, Suzuki T, Suzuki S, Suzuki N, Ogawa K. Sudden Onset Hearing Loss and Vertigo Just Before Posterior Inferior Cerebellar Artery Infarction (Lateral Medulla Syndrome). Otol Neurotol. 2012 Sep 19. [Epub ahead of print]

○Masuda M, Kanzaki S, Minami S, Kikuchi J, Kanzaki J, Sato H, Ogawa K. Correlations of inflammatory biomarkers with the onset and prognosis of idiopathic sudden sensorineural hearing loss. Otol Neurotol. 2012; 33(7):1142-50.

○Oishi N, Shinden S, Kanzaki S, Saito H, Inoue Y, Ogawa K. Effects of tinnitus retraining therapy involving monaural noise generators. Eur Arch Otorhinolaryngol. 2012 Feb 14. [Epub ahead of print]

○Kanzaki S, Saito H, Inoue Y, Ogawa K. A new device for delivering drugs into the inner ear: Otoendoscope with microcatheter. Auris Nasus Larynx. 2011 May 19. [Epub ahead of print]

○Hosoya M, Fujioka M, Matsuda S, Ohba H, Shibata S, Nakagawa F, Watabe T, Wakabayashi K, Saga Y, Ogawa K, Okano HJ, Okano H. Expression and function of Sox21 during mouse cochlea development. Neurochem Res. 2011; 36:1261-9.

○Cui Y, Sun GW, Yamashita D, Kanzaki S, Matsunaga T, Fujii M, Kaga K, Ogawa K. Acoustic overstimulation-induced apoptosis in fibrocytes of the cochlear spiral limbus of mice. Eur Arch Otorhinolaryngol. 2011; 268: 973-8.

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○野口佳裕，伊藤卓，川島慶之，西尾綾子，本田圭司，喜多村 健：前庭水管拡大症を伴うSLC26A4, ATP6V1B1, SIX1変異例の聴平衡覚所見の検討. Equilibrium Res 72 (2) : 97-106, 2013

○Kimura Y, Kubo S, Koda H, Shigemoto K, Sawabe M, Kitamura K: RNA analysis of inner ear cells from formalin fixed paraffin embedded (FFPE) archival human temporal bone section using laser microdissection -A technical report. Hear res 302: 26-31, 2013

○本田圭司，野口佳裕，加藤智史，奥野秀次，喜多村 健：網羅的解析により診断された耳小骨奇形を合併したミトコンドリア3243変異例. Otology Japan 23(3) : 227-32, 2013.

○Nishio A, Noguchi Y, Sato T, Naruse T, Kimura A, Takagi A, Kitamura K: A DFNA5 Mutation Identified in Japanese Families with Autosomal Dominant Hereditary Hearing Loss. Ann Hum Genet (in press)

○Sumi T, Watanabe I, Tsunoda A, Nishio A, Komatsuzaki A, Kitamura K: Longitudinal study of 29 patients with Meniere's disease with follow-up of 10 years or more (In commemoration of Professor Emeritus Isamu Watanabe). Acta Otolaryngol. 2011 Nov 6. [Epub], Acta Otolaryngol 132: 10-5, 2012

○Takahashi N, Tsunoda A, Shirakura S, Kitamura K: Anatomical feature of the middle cranial fossa in fetal periods: possible etiology of superior canal dehiscence syndrome. Acta Otolaryngol. 2011 Dec 27. [Epub], Acta Otolaryngol 132: 385-90, 2012 (4月)

○野口佳裕，高橋正時，籾山直子，杉本太郎，喜多村 健：正円窓小窩，内耳道への進展を認めた迷路内神経鞘腫の1例. 日耳鼻会報 115：687-92, 2012

○岡田隆平，角田篤信，籾山直子，岸根有美，喜多村 健，岸本誠司，秋田恵一：Thiel法による解剖体固定法とその有用性についての検討.日耳鼻会報 115:791-4, 2012

○Kato T, Nishigaki Y, Noguchi Y, Fuku N, Ito T, Mikami E, Kitamura K and Tanaka M: Extended screening for major mitochondrial DNA point mutations in patients with hereditary hearing loss. J Hum Genet, (13 September 2012) | doi:10.1038/jhg.2012.109 [OnlineP]

○Kato T, Fuku N, Noguchi Y, Murakami H, Miyachi M, Kimura Y, Tanaka M and Kitamura K: Mitochondrial DNA haplogroup associated with hereditary hearing loss in a Japanese population. Acta Otolaryngol. 132: 1178-82, 2012

○喜多村 健：特集 急性感音難聴の最新治療戦略 遺伝子からみた急性感音難聴. JOHNS 28(5) : 729-32, 2012