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Middle-Ear Dexamethasone Delivery via Ultrasound Microbubbles Attenuates Noise-Induced Hearing Loss

Cheng-Ping Shih, MD, PhD; Hsin-Chien Chen, MD, PhD; Yi-Chun Lin, MS; Hang-Kang Chen, MS; Hao Wang, MS; Chao-Yin Kuo, MD; Yuan-Yung Lin, MD; Chih-Hung Wang, MD, PhD

Objectives/Hypothesis: In this study, we expanded our previous investigation by testing the efficiency of trans-round window membrane dexamethasone (DEX) delivery mediated by ultrasound (US)-aided microbubbles (MBs) and its preventive effects regarding noise exposure in animal models.

Study Design: Live animal model.

Methods: Forty-two pigmented male guinea pigs were divided into the following three groups: an US-MBs (USM) group, in which the tympanic bulla was filled with DEX and MBs and exposed to US; a round window soaking (RWS) group, without the US irradiation; and a control group. The above-mentioned manipulations were performed 2 hours prior to white noise exposure. The cochlear damage, including auditory threshold shifts, hair cell loss, and expression of cochlear HMGB1, was evaluated.

Results: The enhanced DEX delivery efficiency of the USM group was approximately 2.4× to 11.2× greater than that of the RWS group. After the noise exposure, the RWS group showed significant cochlear protection compared with the control group, and more significant and dominant protective effects were demonstrated in the USM group.

Conclusions: The application of US-MBs provides a safe and more effective approach than spontaneous diffusion, which is commonly used in clinical practice; thus, this technique holds potential for future inner-ear drug delivery.

Key Words: Round window membrane, ultrasound, microbubble, dexamethasone, noise exposure, hearing loss, intratympanic injection, drug delivery.

Level of Evidence: NA

INTRODUCTION

Glucocorticoids are applied clinically for the treatment of various inner-ear disorders and are the primary treatment for idiopathic sudden sensorineural hearing loss (SSNHL) and autoimmune inner-ear disease. Although hearing loss can have multiple causes, immune-mediated inflammation is one underlying mechanism, as indicated by the favorable actions of corticosteroids as treatments for many inner-ear disorders.

Glucocorticoids modulate the inflammatory response by inhibiting tumor necrosis factor-α–induced cytokine secretion from cochlear spiral ligament fibrocytes in vitro, increasing cochlear blood flow in vivo, and enhancing glutathione biosynthesis in the cochlear spiral ganglion. Glucocorticoids, by suppressing cochlear responses to oxidative stress, ischemia, and inflammation, show both therapeutic and preventive effects against various cochlear insults, including endolymphatic hydrops, cochlear implants, autoimmune inner-ear diseases, acoustic trauma, and noise-induced hearing loss (NIHL).

However, systemically administered glucocorticoids can have adverse side effects, including vascular necrosis of the hip, adrenal insufficiency, gastrointestinal hemorrhage, and elevated blood sugar. In the inner ear, the blood-cochlea barrier further limits glucocorticoid effectiveness by preventing penetration of systemically delivered medication. Consequently, drugs for treatment of inner-ear disorders require local delivery by, for example, intratympanic (IT) or intracochlear approaches. The IT approach involves injection of a drug into the middle ear through the tympanic membrane and is less invasive than the intracochlear approach. The delivery efficiency or sustained delivery via the round window membrane (RWM) can be improved using materials and devices such as hydrogels, nanoparticles, MicroWick, microcatheters, and hyaluronic acid liposomal gels.

We previously demonstrated an enhancement of trans-RWM drug delivery by sonophoresis with ultrasound (US)-aided microbubbles (MBs). Therapeutic agents or medications for the cochlea often have limited access to the inner ear via the systemic blood supply because of the blood-cochlea barrier, but this technique increases the RWM permeability and facilitates drug delivery directly into the inner ear. MBs, which...
previously have been used primarily as contrast agents, are air-core bubbles encased in a lipid, protein, or polymer shell and typically have a size range of 1 to 8 μm. Upon exposure to US, MBs can exhibit several physical phenomena, including oscillation, cavitation, or sonoporation, which cause transient and reversible changes in the membrane permeability of surrounding cells. For this reason, MBs are now being intensively investigated for use in US-mediated gene and drug delivery.\textsuperscript{15,16}

Here, we used US-aided MBs (US-MBs) to deliver synthetic glucocorticoid dexamethasone (DEX) into the inner ear of an NIHL guinea pig model to determine the effectiveness of this approach over the spontaneous absorption at the RWM following an IT injection. We examined the delivered concentration of DEX and its effects on hearing threshold shifts, hair cell loss, and inflammation.

**MATERIALS AND METHODS**

**Animals and Study Design**

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan. Forty-two pigmented male guinea pigs (250–350 g) with a normal Preyer reflex were separated into three study groups: 1) a US-MB (USM) group, in which the tympanic bulla was filled with 200 μL of a mixture of DEX (5 mg/mL) and MBs, followed by three consecutive 1-minute US exposures; 2) a round window soaking (RWS) group to simulate human IT procedures, in which the tympanic bulla was filled as above with 200 μL of the DEX/MB mixture but without US treatment; and 3) a control group, in which the tympanic bulla was filled with 200 μL of 0.9% saline (Fig. 1). Two hours later, the animals were subjected to a noise treatment.

**MB Preparation**

SonoVue (Bracco, Milan, Italy) phospholipid MBs were freshly reconstituted prior to use to give a suspension containing 2–5 × 10^8 bubbles/mL with a mean diameter of 5.7 μm (Fig. 2A). Albumin MBs were kindly provided by Dr. AH Liao and prepared as described previously.\textsuperscript{14,17} The albumin MB mean diameter was 7.09 μm and the mean concentration was 2.9 × 10^9/mL (Fig. 2B). Our previous experiments determined that a 10-fold MB dilution, which yields a concentration of approximately 10^8 bubbles/mL, gave the most effective penetration of agarose phantoms when combined with US.\textsuperscript{17} Therefore, 0.9 mL of DEX was mixed with 0.1 mL of albumin MBs.

**US Irradiation**

Irradiation was delivered by two US devices, a ST2000V with a 6-mm-diameter transducer and a KTAC-4000 with a 2-mm-diameter transducer (Nepa Gene, Chiba, Japan). The optimal US exposure settings, determined previously, were as follows: acoustic intensity 3 W/cm² for three consecutive 1-minute courses; duty cycle 50%; and testing frequencies 0.5, 1, 3, and 5 MHz. The transducer was placed 5 mm away from and facing the RWM, and sonication was applied for the indicated duration.

**Surgery**

The guinea pigs were anesthetized with 10 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany) and 80 mg/kg ketamine (Imalgene; Merial, Lyon, France) intramuscularly. A fenestration is made in the RWM with a RWD-055 needle (Nepa Gene, Chiba, Japan). The RWD-055 needle is filled with 0.9 mL of DEX and 0.1 mL of albumin MBs, followed by three consecutive 1-minute US exposures. Two hours later, the animals were subjected to a noise treatment.

![Fig. 1. Study design flow chart. ABR = auditory brainstem responses; DEX = dexamethasone; ELISA = enzyme-linked immunosorbent assay; HMGB1 = high mobility group box 1; IHC = immunohistochemistry; NIHL = noise-induced hearing loss; RWS = round window membrane soaking treatment; USM = ultrasound microbubble treatment.](image-url)
(approximately 4 mm in diameter) was created in the tympanic bulla under an operating microscope (P-170; Carl Zeiss, Jena, Germany) to expose the round window and allow infiltration of the DEX/MB mixture and US irradiation (Fig. 3). Following the US irradiation, the mixture was removed and the tympanic bulla irrigated with sterile physiological saline. The surgical wound was sutured in layers.

The concentration of DEX delivered to the cochlear perilymph was assessed after euthanizing the animals with CO₂ gas. A 10-μL pipette microtip was gently inserted through a cochleostomy inferior to the RWM to aspirate perilymphatic fluid. The collected samples were immediately centrifuged and stored at −80 °C until used for fluorescence analysis.

**Noise Exposure**

The guinea pigs were anesthetized, placed in a soundproof booth with a loudspeaker (V12 HP; Tannoy Ltd., United Kingdom) mounted above the center of the cage, and exposed to white noise at a 118-dB sound pressure level for 5 hours.

**Enzyme-Linked Immunosorbent Assay Analysis of DEX Levels**

A commercial competitive enzyme-linked immunosorbent assay (ELISA) kit was used for DEX determination (Neogen Corp., Lansing, MI). The plate was read using an ELISA microplate reader equipped with a 650-nm filter (Synergy H4 Hybrid Reader; BioTek Instruments, Winooski, VT). The minimal
The detectable DEX concentration in the cochlear perilymph samples was 3 ng/mL.

Cochlear Surface Preparations and Hair Cell Survival

After the noise treatment, the animals were transcardially perfused with 4% paraformaldehyde. The cochleae were removed and the cochlear lateral wall and Reissner’s membrane were excised. The remainder of the cochlea was permeabilized with 0.3% Triton X-100, stained with 2% Alexa Fluor 488-conjugated phalloidin (Molecular Probes/ThermoFisher Scientific, Waltham, MA), and incubated with 5 mg/mL 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes/ThermoFisher Scientific). The entire length of the flat surface preparation of the organ of Corti was examined with a Leica DMI6000B inverted microscope (Leica Microsystems, Wetzlar, Germany).

Outer hair cells’ (OHCs) survival rates were calculated using the following formula: OHC survival rate% = 100 × [(the number of OHCs present in the examined specimens) / (the number of examined specimens) / 3].18

Cochlear Cryosections

Paraformaldehyde (4% in phosphate-buffered saline) was gently perfused into the cochleae. After a 2-hour post-fixation, the cochleae were decalcified in 10% ethylene diamine tetraacetic acid at 4 °C under rotation and then immersed in graded sucrose solutions (15%, 20%, and 25%) for 30 minutes, followed by overnight immersion in 30% sucrose at 4 °C under rotation. The cochleae were then transferred into optimal cutting temperature compound, frozen, and cut with a cryostat microtome into 10- to 14-μm mid-modiolar sections, which were mounted on glass slides.

Immunohistochemistry

The Mouse/Rabbit PolyDetector HRP/DAB Detection System (Bio SB Inc., Santa Barbara, CA) was used for immunohistochemical staining. The slides were covered with mouse anti–HMGB-1 (1:50; Novus Biologicals, Littleton, CO) or anti–intercellular adhesion molecule-1 (ICAM-1; 1:50; eBioscience, Inc., San Diego, CA) primary antibodies. The slides were counterstained with hematoxylin, dehydrated in a graded alcohol series, cleared in xylene, mounted in Permount (Fisher Scientific, Pittsburgh, PA), and examined with an Olympus BX50 microscope (Olympus Life Science, Waltham, MA).

Quantification of Immunohistochemical Staining

Images were analyzed using the open-source Fiji software (ImageJ; https://fiji.sc/).21 DAB (3,3′-diaminobenzidine) staining intensities were multiplied by the number of pixels representing the selected immunostaining area, expressed in arbitrary units (AU) for the different cochlear cell types, and subjected to histogram analysis.

Auditory Brainstem Response Recording

Auditory function was assessed by recording the auditory brainstem responses, as previously described.20 Specific stimuli (clicks and 8-, 16-, and 32-kHz tone bursts) were generated using SigGen software (Tucker-Davis Technologies, Gainesville, FL).

Statistical Analysis

Statistical analysis was performed using a two-tailed Student t test. Results are expressed as the mean ± standard error of the mean. Differences were considered significant at P < .05.

<table>
<thead>
<tr>
<th>MB</th>
<th>US Frequency, MHz</th>
<th>Fold Increase of Transmembrane Inner-Ear Delivery (Relative to Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SonoVue</td>
<td>0.5</td>
<td>3.5</td>
</tr>
<tr>
<td>SonoVue</td>
<td>3.0</td>
<td>11.2</td>
</tr>
<tr>
<td>SonoVue</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Albumin-shelled</td>
<td>0.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Albumin-shelled</td>
<td>1.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Albumin-shelled</td>
<td>5.0</td>
<td>3.3</td>
</tr>
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</table>

Efficiency was defined as the fold increase in the delivered DEX concentration using a US-MB approach versus a control group without US irradiation.

DEX = dexamethasone; MB = microbubble; US = ultrasound.

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RESULTS

Figure 4A shows that the inner-ear DEX concentration was significantly higher in the USM groups following SonoVue MB/US treatment at frequencies of 0.5 MHz (1459 ± 233.0 ng/mL), 3 MHz (4631 ± 1002.8 ng/mL), and 5 MHz (1229 ± 410.1 ng/mL) than in the RWS group (414 ± 140.0 ng/mL). The perilymphatic DEX levels were higher in the USM group exposed to 3 MHz than in the groups exposed to 0.5 MHz (P = .006) and 5 MHz (P = .006).

Albumin MBs also significantly increased the perilymphatic DEX levels in the USM group at US frequency settings of 0.5, 1, and 5 MHz (1007 ± 51.6 ng/mL [P = .002], 3271 ± 857.1 ng/mL [P = .005], and 1376 ± 392.3 ng/mL [P = .016], respectively) when compared with the RWS group (414 ± 140.0 ng/mL; Fig. 4B). The DEX delivery was significantly higher for a US frequency of 1 MHz than for 0.5 MHz (P = .01) or 5 MHz (P = .025). We also examined whether the MBs could affect the permeability of RWM in the absence of US treatment. We found no significant difference in delivery efficiency when the RWM was soaked with DEX alone or soaked with DEX combined with MBs (RWS group; 418 ± 125.5 ng/mL vs. 414 ± 140.0 ng/mL; P = .975). These results suggest that the presence of MBs alone would not enhance the permeability of the RWM; this enhancement is only observed when the MBs are used in conjunction with US.

DEX delivery was increased by approximately 3.0- to 11.2-fold with SonoVue MBs and 2.4- to 7.9-fold with albumin MBs when compared with the control (Table I). We used SonoVue MBs for all subsequent experiments.

On days 7, 14, and 28 post–noise exposure, the responses to a click and 8, 16, and 32 kHz stimuli differed significantly between the USM group and the control group (Fig. 5). Conversely, although the RWS group also presented fewer noise-induced threshold shifts compared with the control group, significant differences were only observed following the 8-kHz stimulus on day 28 post–noise exposure. Therefore, the US-aided MB DEX treatment showed superior protective effects against NIHL.

Figure 6 shows the severity of cochlear hair cell loss 28 days post–noise exposure. We used phalloidin staining of the stereociliary bundles to aid in identifying the hair cells, as well as DAPI staining of the nuclei of the OHCs to confirm hair cell loss. The control group showed a prominent loss of OHCs from the organ of Corti, whereas the RWS showed a less prominent loss. The USM group showed the preservation of considerable numbers of OHCs in the basal and second turns, indicating less damage to the cochleae after noise exposure.

Figure 7A shows intense high mobility group box 1 (HMGB1) staining in the spiral ligament, spiral limbus, and spiral ganglion in the control cochleae, but a significant decrease in expression in these regions in the RWS and USM groups. Expression in the control group spiral ligament was especially evident in type I, II, and III fibrocytes. By contrast, the RWS group showed staining in only a few type I and II fibrocytes, whereas the USM group showed no significant positive staining of fibrocytes.

The expression of HMGB1 in all three cochlear regions was statistically different in the control and USM groups (P < .001) and in the control and RWS groups (P < .05), suggesting that the increased expression of cochlear HMGB1 due to acoustic trauma was repressed by the local delivery of DEX (Fig. 7B) and that the US-aided MB treatment was more effective than the RWS treatment for DEX delivery. The expression of ICAM-1, a
cylindrical mitochondria and well-developed rough endoplasmic reticulum and Golgi complexes that can support active transport.25 The middle connective layer comprises fibroblasts, collagen, elastic fibers, and vessels and is the principal site of aging- and inflammation-related thickness changes in the RWM.27,28 The inner epithelial layer contains squamous cells and lacks continuity with the basement membrane, suggesting possible transit of substances between the inner and middle ear. The RWM permeability is affected by several factors, including the constitutive structural characteristics of the membrane; the delivered particle size, concentration, liposolubility, and electrical charge; and the travel path.25,26 Substance transport across the RWM can involve several cellular processes: diffusion down a concentration gradient, pinocytosis, or transcellular movement through channels.25,26 The outer epithelial layer of the RWM plays a decisive role in controlling transmembrane transport.25,29

The permeability of the RWM was clearly enhanced by application of US-aided MBs. The addition of US energy transforms the MBs into cavitation-enhancing

Heinrich et al. showed that a pretreatment with DEX could prevent noise-induced glucocorticoid receptor degradation in the lateral wall of the cochlea,9 implying that DEX plays a role in receptor protein turnover and restoration. However, animal studies using IT DEX treatment for NIHL have shown inconsistent results,9,21,22 possibly reflecting differences in the degree of acoustic trauma sustained in each animal model, the applied dose, and the duration of the DEX administration.

IT DEX administration causes a dose-dependent preservation of retrocochlear auditory neurons in an NIHL mouse model.22 Glucocorticoids can be used as a primary initial treatment or as a salvage therapy for SSNHL, but the clinical therapeutic outcomes of IT steroid treatments are associated with the application timing, dosage, and administration protocol.22,24 Regardless, the key factor is delivery of a sufficient DEX concentration to the perilymph.

Any medications prepared in solution form and infused into the middle ear have only short residence times to contact the RWM because of drainage through the Eustachian tube. Consequently, clinical applications of IT corticosteroids may require repeated injections, avoidance of swallowing by the patient, placing the patient's head slightly lower than the body, or instructing the patient to lie in a supine position with the head turned 45 degrees toward the contralateral side for various durations. These ambiguous factors result in varying therapeutic responses, but IT therapies are increasingly used in clinical practice for the treatment of several hearing disorders.

US-aided MB treatment in the RWM is a feasible, safe, and highly efficient approach for treating ear disorders.14 The RWM is a triple-layered structure comprising the outer epithelial layer, middle connective tissue layer, and inner mesothelial layers.25,26 The cuboidal cells lining the outer epithelial layer have extensive lateral interdigitations between neighboring cells and tight junctions at their surfaces. Their cytoplasm contains abundant cylindrical mitochondria and well-developed rough endoplasmic reticulum and Golgi complexes that can support high rates of active transport.25 The middle connective layer comprises fibroblasts, collagen, elastic fibers, and vessels and is the principal site of aging- and inflammation-related thickness changes in the RWM.27,28

The inner epithelial layer contains squamous cells and lacks continuity with the basement membrane, suggesting possible transit of substances between the inner and middle ear. The RWM permeability is affected by several factors, including the constitutive structural characteristics of the membrane; the delivered particle size, concentration, liposolubility, and electrical charge; and the travel path.25,26 Substance transport across the RWM can involve several cellular processes: diffusion down a concentration gradient, pinocytosis, or transcellular movement through channels.25,26 The outer epithelial layer of the RWM plays a decisive role in controlling transmembrane transport.25,29

The permeability of the RWM was clearly enhanced by application of US-aided MBs. The addition of US energy transforms the MBs into cavitation-enhancing
mediators that concentrate the energy of relatively noninteractive pressure waves to permeabilize the cell membrane.\textsuperscript{16} Both stable and inertial cavitation effects can be initiated by the irradiated MBs, depending on their chemical constitution. Stable cavitation creates microstreaming around the MBs, whereas inertial cavitation produces a shock wave. Moreover, an asymmetrical collapse of the MBs can result in sonic-speed microinjection at the cell surface, creating transient but nonlethal micropores in the cell membrane that promote drug transit.\textsuperscript{30} Here, the US-aided MBs effectively facilitated the transmembrane delivery of DEX into the inner ear. Using this intervention, the efficiency of DEX delivery was approximately 2.4× to 11.2× greater than that obtained with the current clinical treatment, namely soaking DEX around the RWM. The technique had no side effects on hearing, indicating its potential for clinical use in inner-ear drug and gene delivery.

Wang et al. have implicated HMGB1, a marker of inflammation, in endotoxin-related lethality in mice.\textsuperscript{31} Inflammation has been implicated in NIHL, so we selected HMGB1 expression to examine the effects of DEX on the inflammatory responses in our NIHL model. HMGB1 can be passively released from necrotic cells or actively secreted from activated immune cells into the cytoplasm and the extracellular space.\textsuperscript{32–34} It serves as an endogenous danger-signaling molecule, termed alarmin, and as a proinflammatory mediator that induces the production of proinflammatory cytokines and chemokines by interacting with toll-like receptors and the receptor for advanced glycation end products. Previous studies have shown HMGB1 to suppress inflammation; however, current evidence indicates that HMGB1 mediates vital organ injury, suggesting a role as a damage-associated molecular pattern molecule.\textsuperscript{34} Our guinea pig NIHL model showed a marked increase in cochlear HMGB1 expression on day 4 after the noise exposure, and the main expression site was the spiral ligament of the lateral wall—a common site of cochlear inflammation. A DEX pretreatment significantly reduced cochlear HMGB1 expression and alleviated the experimental noise-induced hearing threshold shifts and OHC loss.

Effective interventions to reduce NIHL mainly rely on hearing-conservation programs and use of hearing-protection devices. However, improvements in these interventions and in the development of preventive drugs remain major issues. The US-aided MBs both permeabilize the RWM and maintain a prolonged membrane

Fig. 7. Pretreatment with DEX suppresses the expression of HMGB1 and ICAM-1 in noise-exposed cochlea. (A) Representative HMGB1 staining of cochlear basal turn sections assessed on day 4 after the noise exposure of the control, RWS, and USM groups. (B) HMGB1-specific staining intensities shown in AUs (mean values ± SEM) in the spiral ligament, spiral limbus, and spiral ganglion cells in the three experimental groups. *P < .05, **P < .01, ***P < .005. I, II, III, IV = location for classification of fibrocyte types I–IV. Scale bars = 100 μm. AU = arbitrary units; DEX = dexamethasone; HMGB1 = high mobility group box 1; ICAM-1 = intercellular adhesion molecule-1; RWS = round window membrane soaking treatment; SEM = standard error of the mean; USM = ultrasound microbubble treatment.
CONCLUSION

The administration of DEX using a trans-RWM approach showed significant protective effects against noise-exposure injury in the NIHL guinea pig model, as indicated by smaller hearing threshold shifts, greater preservation of OHCs, and abatement of inflammation-related HMGB1 expression. The US-aided MB technique can increase the concentration of DEX in the perilymph, thereby achieving safer and more effective therapeutic outcomes in preventing NIHL than are obtained with common clinical protocols that rely on spontaneous diffusion. We believe that the US-aided MB technique holds potential for future applications and clinical translation for inner-ear drug delivery.

Acknowledgments

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