Intracochlear Distribution of Intra-tympanically Injected QDDNs in Rats and Analysis of the Hearing Recovery According to the Frequency in SSNHL

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Intratympanic dexamethasone (IT-DEX) injection is a useful treatment option for patients that fail systemic steroid therapy for treatment of sudden idiopathic sensorineural hearing loss (SSNHL). The clinical results of patients with SSNHL treated with IT-DEX are not always consistent with the apical-basal gradient pattern of concentration, even though it has been generally known that the treatment of SSNHL is dependent on the concentration of DEX. Unfortunately, there have been no reports of a tendency for improved hearing gains at high frequencies (basal turn). Therefore, we investigated the distribution pattern of DEX in the cochlea with the assistance of quantum dot-dexamethasone nanocomplex (QDDN). QDDNs were injected intratympanically into the bulla of Sprague-Dawley rats and the distribution of the QDDN in the cochlea was analyzed by fluorescence microscopy. From our in vivo animal study and statistical comparisons of clinical data, it was clearly observed that the concentration distribution of QDDN at the basal turn corresponds with the hearing improvement in patients with SSNHL, especially at 8000 Hz. These results of animal research are useful for further applications for treatment of human patients for SSNHL and are effective to determine the optimized dosages of DEX.

Key words: Quantum dots - dexamethasone nanocomplex (QDDN), Intracochlear Distribution, Sensorineural hearing loss (SSNHL)

Introduction

Steroids are the mainstay for treatment of sudden sensorineural hearing loss (SSNHL) due to their anti-inflammatory effects on cochlear pathology associated with hearing loss.¹,² Although steroid treatment is the most effective treatment option to date, no difference has been detected between steroid and placebo treatments for hearing loss.³ While the exact mechanisms involved in steroid distribution throughout the cochlear region and their mode of action have not yet been established, direct injection of drugs into the tympanic cavity is widely used in patients with SSNHL and Meniere’s Disease, when oral steroids and medications fail or are contraindicated due to co-morbidities. In general, 3 treatment procedures are adopted for inner ear perfusion through the round window membrane (RWM) from the middle ear. In the first method, the entire middle ear cavity is filled with fluid, which is subsequently absorbed through the RWM.⁵ The second method uses a microtubule or microwick in the RWM for direct and continuous infusion into the cochlea,⁶ and the third method places a drug-soaked gel foam into the RWM niche, where it is absorbed passively into the inner ear.⁷ All of these methods can deliver drugs to the cochlea effectively. However, the concentration of the delivered drug is dependent on the diffusion coefficient of the drug through the RWM, e.g., a high concentration at the basal turn, but a low concentration or failure of delivery of the drug at the apical turn (so called apical-basal gradient).⁸ Therefore, the effect of intratympanic steroid injection (ITSI) in patients with SSNHL might be better for high tone areas (e.g., at the basal turn) than for low tone areas (e.g., at the apical turn). However, most clinical studies of interfrequency differences after ITSI indicated similar findings between high and low hearing frequencies,⁹,¹² and better hearing recovery at 250 Hz was found in 1 study.¹³ The mechanisms of intracochlear distribution and action of ITSI are unknown, although it is plausible that good hearing recovery at low frequencies indicate that a sufficient amount of injected drug was delivered to the apical turn.

In this study, we used quantum dot-dexamethasone nanocomplexes (QDDNs) to determine the anatomical distribution of intratympanically injected dexamethasone and to determine the intracochlear distribution of the drug. There are many advantages to the use of QDs as fluorophores compared to
the use of classic fluorescence staining methods. For example, nanoparticles are water soluble and can be used for in vivo experiments. The fluorescence potency is 20 times stronger than organic molecules and is stable for up to 1 year. The QDDNs easily diffuse into the cochlea, reflect the distribution of steroid medication in the inner ear, and remains stably expressed after 24 h. The purpose of this study was to determine the actual distribution of injected steroid through the tympanic membrane of rats, in the form of QDDNs. Furthermore, the animal experimental data obtained from the intracochlear distribution of QDDNs was indirectly compared with clinical data from patients with SSNHL treated by IT-DEX injection.

Materials and Methods

Preparation of the QDDNs

Adipic acid dihydrazide (0.25 M) and imidazole (0.1 M) were melted into 1 mL of phosphate buffer solution (PBS, pH 7.4), subsequently, 4 mg of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC) was added to this mixture and stirred for 30 min. After reaction, this solution (solution A) was centrifuged at 500 rpm for purification and removing impurity. One milliliter of DEX was added to solution A and then mixed together for 2 hrs at 4°C to combine between amine group of Adipic acid dihydrazide and carboxyl group of DEX. After this chemical reaction, 2 mg of EDC was added to mixture and stirred 30 min again. Finally, QDs were added to above mixture and stirred 30 min to produce QDDNs by EDC chemistry. Final compound was stored at 4°C. Although unknown pharmacological effects may occur through bonding of QDs and DEXs, it does not influence our experiments because the main purpose of QDDN synthesis was to characterize the concentration gradients by fluorescence microscopy.

In vivo intratympanic injection of QDDNs in rats and their characterizations

Twelve Sprague-Dawley rats aged 30 days, weighing 120-150 g were used. The rats were divided into 9 experimental (18 ears) and 3 control groups (6 ears). Each experimental group was divided into 3 subgroups according to the time interval after intratympanic injection of the QDDNs. A mixture of ketamine (40 mg/kg) and xylazine (4 mg/kg) was injected intraperitoneally to induce anesthesia. The bulla and tympanic membrane were exposed through a retroauricular incision and 20 µL of QDDN was injected into the bulla through the tympanic membrane under microscopic guidance. Three rats were sequentially sacrificed 1, 2, and 24 h after injection. The euthanized rats were perfused with 4% paraformaldehyde (PFA) solution and the cochleae were harvested and fixed in 4% PFA solution for an additional 6 h. After a decalcification procedure

Figure 1. Characteristics of Quantum Dot-Dexamethasone Nanocomplex. (A) The chemical formula of dexamethasone, (B) the solution of conjugated Quantum Dot-Dexamethasone Nanocomplex (QDDN), (C) and the absorbance/fluorescence histogram of the QDDN.
in EDTA solution for 1 week, 10 to 30% sucrose solutions were perfused into the cochleae from low to high concentrations over 2.5 h and stored at a 30% solution overnight. The cochleae were perfused and stored in optimal cutting temperature (OCT) solution for 1 day and then frozen in a cryomold for cutting. Cutting was performed with a Cryostat (CM 1510S-3, Leica, Wetzglar, Germany) at 7-µm thickness and images were obtained using an immunofluorescence microscope (BE/DM-IRB, Leica, Wetzglar, Germany). The animal protocol used in this study was reviewed and approved by the Pusan National University Institutional Animal Care and Use Committee (PNU-IACUC).

Data collection and analysis of patients with SSNHL
Clinical data was collected by retrospective chart review of 137 patients with SSNHL admitted to Pusan National University Hospital and treated with steroids beginning January 1, 2000. The subjects were divided into 3 groups according to the treatment methods: Group A was treated with systemic steroids (SS), Group B was treated with SS plus intratympanic steroid injections (ITSI), and Group C was treated with ITSI only. The pure tone audiogram results were compared before the treatment, and 3 months after the treatment in each group. Sixty age- and gender-matched patients in each of group A and group B were enrolled in this study. Seventeen patients with diabetes mellitus or other systemic diseases were selected for group C. Systemic steroid therapy was started with 80 mg of prednisolone and the dose was tapered over 14 days. Intratympanic injections were performed using 0.3 cc of dexamethasone (5 mg/mL) daily for the first week and alternate days for the second week. The degree of hearing recovery was determined by Siegel’s criteria; complete, partial, and mild recovery were considered as recovered hearing.14 Hearing recovery was analyzed according to hearing frequency at 8 pitches from 125 to 8000 Hz. The results of the overall hearing recovery of each group were compared statistically using Fisher’s exact test. Hearing recovery according to frequency was analyzed by the Tukey method.

Results and Discussion

Observation of intracochlear distribution of the QDDN
The mainstay of treatment for patients with SSNHL is steroid therapy by oral or intravenous routes.12,15,16 Transtympanic drug delivery to the inner ear is currently a commonly used treatment option. The drug in the middle ear diffuses into the inner ear through the RWM, according to the diffusion coefficient of the drug. Intracochlear drug delivery reaches high concentrations in the basal turn and low concentrations in the apical turn (apical-base gradient).17 We observed similar results using fluorescence imaging of QDDNs in the animal study. The QDDN was found in the entire cochlea from base to apex 1 h after intratympanic injection. High fluorescence signaling was observed at the basal and middle turn of the cochlea in the spiral ganglion cells (arrow a), spiral ligament (arrow b), and organ of Corti (arrow c). In order of concentration expression, the spiral ganglion neurons (SGNs), hair cells (HCs), and spiral ligaments (SLs) were observed (Figure 2(A)). That is, the DEXs were highly expressed in the SGNs at the basal and middle turn of the cochlea and were relatively lower in the SLs of the apical turn. The QDDN was not expressed in SLs after 2 h, and was weakly expressed in HCs at the basal turn only. The QDDN in the SGNs were highly expressed compared to the HCs and SLs at 2 h (Figure 2(B)). In the fluorescence images taken 24 h after injection, QDDN was not observed at the HCs and SLs but was expressed in parts of the SGNs (Figure 2(C)).
The steroids injected through the RWM are absorbed into the cochlea according to the distribution of glucocorticoid receptors in the inner ear. The effects of steroids on the inner ear are nonspecific and are likely associated with anti-inflammatory or anti-immune responses of the entire cochlea with specific direct effects on inner ear cells, especially the SGNs, HCs, and SLs. The injected dexamethasone (QDDN) in this study diffused into the SGNs, HCs, and SLs in 1 h, similar to the study reported by Rarey et al. The intracochlear distribution was weaker at the apical turn than at the basal and middle turn. These results are consistent with the cochlear simulator program as described above.

**Result of steroid treatment in patients with SSNHL**

Figure 3(A) shows the comparison of hearing improvement at various frequencies with systemic steroids (SS, Group A, blue bars), systemic steroids combined with intratympanic dexamethasone (SS+ITSI, Group B, red bars), and intratympanic dexamethasone injection only (ITSI, Group C, green bars). The rate of hearing recovery as determined by Siegel’s criteria was 66.7% in group A, 65.0% in group B, and 64.7% in Group C, respectively. The rate difference among groups was not statistically significant (Table 1). However, hearing recovery at 8000 Hz in Group A was significantly lower compared to the recovery at the other frequencies (Tukey, p < 0.05) (Figure 3(A)). The hearing recovery according to frequency was not different from Group B and C (Figure 3(A)). According to the frequency, hearing improvement was significantly better in group A than group B at 8000 Hz, which indicates high concentration at the basal turn (Figure 3(B)).

The cochlear fluid simulator program developed at Washington University showed that the drug in the rat cochlea takes more than 24 h to reach the apical turn of the cochlea through the RWM. Therefore, the drug concentration in the apical turn may not be sufficient to provide the treatment effects observed at the basal turn. These differences in drug concentrations between the apical and basal turn could affect the treatment results of intratympanic injection of steroids in patients with SSNHL. Therefore, the treatment effects at high frequencies (basal turn with high drug concentration) might be better than the effects at low frequencies (apical turn with low drug concentration).

Figure 3(B) shows hearing improvement according to frequency comparisons between treatment systemic steroid (Group A) and oral steroid with intratympanic dexamethasone (Group B) groups. The hearing gain at 8000 Hz was significantly better in Group B (19.9 dB) than in group A (13.7 dB) (Tukey’s test, p < 0.05) (Figure 3(B)).

Many studies on intratympanic steroid injections for the treatment of SSNHL have demonstrated no differences in hearing results at different frequencies. In one report, the hearing recovery was better at 250 Hz, at the apical turn, than at other frequencies. However, another report showed good hearing recovery at 1500 and 3000 Hz in patients with high frequency hearing loss. These reports indicate that the exact mechanism of diffusion and distribution of the drug through the RWM has not been established. The interval for intratympanic injections varies with each reported study; once per week for 3 weeks; 4-5 times over 15 days; and on just one occasion. Hearing was recovered regardless of the injection methods. In this study, we injected the medication once per day for 1 week and then once every 2 days for the next week.

Patients with SSNHL showed better hearing recovery at the basal turn, at 8000 Hz, which is consistent with the results of the experiments on the rat cochlea in this study. Additional study of the most effective concentration of the drug for each site is needed to determine the appropriate dose of the intratympanic injection and the duration of treatment in the clinical setting. Our result of better hearing recovery at 8000 Hz in group B (SS+ITSI group) compared to group A supports the theory of an apical-base concentration gradient in the cochlea proposed by Plontke. This result supports intratympanic steroid injection therapy as a better option for the treatment of high tone SSNHL or noise trauma. Intracochlear steroid distribution after intratympanic injection was evaluated.

**Table 1. Hearing recovery rates of respective groups by Siegel’s criteria**

<table>
<thead>
<tr>
<th></th>
<th>Group A (N=60)</th>
<th>Group B (N=60)</th>
<th>Group C (N=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete recovery</td>
<td>16</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Partial recovery</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Slight improvement</td>
<td>16</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>No improvement</td>
<td>20</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>Hearing improvement (%)</td>
<td>40(66.7)</td>
<td>39(65.0)</td>
<td>11(64.7)</td>
</tr>
</tbody>
</table>

Group A: Systemic steroid group, p > 0.05
Group B: Systemic steroid with Intratympanic dexamethasone group
Group C: Intratympanic dexamethasone group

![Figure 3](image-url)

Figure 3. (A) Comparison of hearing improvement according to various frequencies with systemic steroids (group A, blue bars), systemic steroids combined with intratympanic dexamethasone (group B, red bars), and intratympanic dexamethasone injection only (group C, green bars). (B) Hearing improvement according to frequency comparison between systemic steroid (Group A) and oral steroid with intratympanic dexamethasone (Group B) groups.
using QDDNs. Steroid medication was distributed according to the apical-base gradient and hearing recovery at 8000 Hz was better than at other frequencies. Intratympanic steroid injection might be successfully used especially in cases with high tone SSNHL.

Conclusion

QDDNs were used for determining the anatomical distribution of intratympanically injected dexamethasone in rats. Then, the experimental data of intracochlear distribution of QDDNs was indirectly compared with clinical data from patients with SSNHL treated by IT-DEX injection. From our in vivo animal studies and statistical comparison of our data with clinical data, it was clearly observed that the concentration distribution of QDDN at the basal turn corresponded to hearing improvement in patients with SSNL, especially at 8000 Hz. This work provides an initial estimate of drug delivery through the RWM and via systematic comparisons between animal studies and clinical data, provides direction for future clinical studies.

References

18. Cochlear Fluids Simulator V 1.6h from http://oto2.wustl.edu/cochlea/model.htm