

Article

Evaluation of Intratympanic Alpha-Lipoic Acid and Diltiazem as Alternatives to Dexamethasone in Noise-Induced Hearing Loss in a Murine Model

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Abstract

This study evaluates the protective effects of alpha-lipoic acid (ALA), diltiazem (DIL), and N-acetylcysteine (NAC) as potential adjunctive agents to enhance intratympanic dexamethasone (IT-DEX) therapy in noise-induced hearing loss. A two-phase experiment using C57BL/6J mice was conducted. In phase 1, candidate drugs were screened by perilymph concentration analysis using ultra-high-performance liquid chromatography, auditory brainstem response (ABR) threshold, and organ of Corti (OC) morphology. Western blot analysis evaluated inflammatory markers. Phase 2 investigated the synergistic effects of co-administration of the most promising candidates with DEX. All drugs successfully penetrated the inner ear via IT injection. In the noise-induced hearing loss model, ALA and DIL individually demonstrated significant improvements in ABR thresholds and OC morphology compared to DEX alone, while NAC showed no therapeutic benefit. Western blot analysis revealed that ALA and DIL suppressed inflammatory markers through distinct antioxidant-mediated mechanisms, contrasting with DEX's anti-inflammatory pathway. However, combination therapy with DEX + ALA or DEX + DIL increased middle ear inflammation and failed to produce synergistic therapeutic effects. While ALA and DIL showed individual therapeutic promise through complementary mechanisms, combination with DEX did not enhance efficacy, suggesting that simple drug combinations may not translate to improved IT therapy outcomes.

Keywords: noise-induced hearing loss; dexamethasone; alpha-lipoic acid; diltiazem; N-acetylcysteine



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1. Introduction

The inner ear resides within a bony encasement with limited vascular supply and is shielded by the blood-labyrinth barrier, presenting significant challenges for drug penetration [1,2]. Only minimal quantities of systemically administered drugs can permeate this region, prompting extensive research into targeted inner-ear drug-delivery strategies [3,4]. Among various approaches, intratympanic (IT) injection is now the most prevalent. This technique delivers drugs efficiently to the inner ear of an animal model with minimal invasiveness at relatively high concentrations compared with systemic administration [5].

Steroids are the preferred therapeutic agents for managing acute disorders of the inner ear. Dexamethasone (DEX), a long-acting glucocorticoid steroid, is particularly prominent in IT injections and is commonly used to treat Meniere's disease, autoimmune inner ear disease, and idiopathic sudden sensorineural hearing loss (ISSNHL) [6,7]. Despite theoretical expectations of superior inner ear penetration and efficacy with IT-DEX compared with systemic dexamethasone, clinical outcomes in acute inner ear conditions have revealed comparable therapeutic results [8]. Consequently, researchers have been motivated to explore strategies for enhancing the therapeutic potential of IT-DEX.

One promising approach involves identifying pharmacological agents that either surpass the efficacy of DEX or demonstrate synergistic effects when combined. A notable limitation of IT therapy is that it typically involves the administration of a single therapeutic agent, contrasting with the capacity of systemic therapy for multi-drug combinatorial treatments that can potentially augment therapeutic outcomes [9].

Numerous compounds have been investigated for IT delivery due to their potential to mitigate acute damage to the inner ear [10]. Among these, alpha-lipoic acid (ALA), *N*-acetylcysteine (NAC), and diltiazem (DIL) have been reported to exert protective effects on the inner ear when administered via the IT route [10–12]. Although these agents differ in their primary pharmacological classifications—ALA as a direct reactive oxygen species (ROS) scavenger, NAC as a glutathione precursor, and DIL as a calcium channel blocker—their therapeutic mechanisms in the context of cochlear injury converge primarily on antioxidant-mediated protection [11,13–17]. These agents were therefore selected to evaluate whether antioxidant-driven approaches through different upstream pathways could complement or surpass the glucocorticoid-mediated anti-inflammatory efficacy of DEX IT therapy. The lack of direct comparative studies of these agents' therapeutic effects and systematic evaluations of their potential synergistic interactions with dexamethasone is a critical research gap. This study was designed to comprehensively compare drugs previously reported to exhibit protective effects on the inner ear when delivered via the IT route and to assess their potential synergistic effects when co-administered with DEX in a mouse model of noise-induced hearing loss.

2. Materials and Methods

2.1. Drug Selection

The selection of candidate drugs for our experimental investigation was conducted through a comprehensive literature review, applying stringent criteria to identify suitable pharmacological agents. The selection process was guided by three primary criteria: (1) demonstration of protective or restorative effects on the inner ear when administered via IT injection, (2) availability in a liquid formulation without the use of alternative delivery vehicles such as gels or nanoparticles, and (3) status as an approved clinical drug rather than an investigational compound.

Based on these criteria, three drugs were selected for study, with their concentrations determined from previously published experimental data: ALA (0.75 mg/10 μ L), DIL (1 mg/mL), and NAC (20 mg/mL) [10–12]. A sham control group received normal saline, and a DEX (5 mg/mL) group was included for comparative analysis as a standard IT injectable treatment.

2.2. Animal Preparation and Noise Exposure

Seven-week-old male C57BL/6J mice were purchased from Orient Bio (Sunngam, Republic of Korea). Eight mice were randomly allocated to each experimental group using a simple randomization method. No specific strategies were employed to control for

potential confounders such as the order of treatments or cage location. Animals that died during the experimental procedures or the housing period were excluded from the analysis.

Mice were housed in a specific pathogen-free (SPF) facility under controlled environmental conditions (temperature: 22 ± 2 °C, humidity: $50 \pm 10\%$, 12 h light/dark cycle). Animals were group-housed (4–5 mice per cage) in individually ventilated cages (IVCs) with autoclaved bedding and had ad libitum access to standard rodent chow and filtered water. No environmental enrichment was provided.

In the mouse model of noise-induced hearing loss, 110 dB sound pressure level (SPL) white noise centered at 10 kHz was administered for 60 min. White noise was generated using random noise generators (B&K Type 1027, Bruel & Kjaer, Nærum, Denmark) placed on top of a specially designed acrylic box (53 cm $\times$ 35 cm $\times$ 53 cm $\times$ 2 cm). The box contained eight pie-shaped wire-mesh cage compartments, with a single mouse housed in each compartment to ensure uniform and individual noise exposure [14].

2.3. Experimental Design

The study was conducted in two distinct experimental phases. The first phase was designed to identify the most effective candidate drug by rigorously evaluating drug distribution and therapeutic potential. To confirm the successful delivery and penetration of IT-administered drugs to the inner ear, seven-week-old male C57BL/6J mice underwent simultaneous IT and intraperitoneal (IP) injections. Perilymph sampling was performed 15 min post-injection, with subsequent analysis using ultra-high-performance liquid chromatography (uHPLC). Drug efficacy was comprehensively assessed using a noise-induced hearing loss mouse model. Evaluation metrics included the auditory brainstem response (ABR) threshold and a morphological assessment of the organ of Corti (OC) through hematoxylin and eosin staining. Following initial screening, the most promising drugs underwent mechanistic investigation through immunofluorescence and Western blot analyses, focusing on key inflammatory markers (nuclear factor kappa-B [NF- κ B], tumor necrosis factor alpha [TNF- α], and interleukin [IL]-1 β and IL-6) in comparison with DEX.

Based on the predefined screening criteria of Phase 1, candidate agents were advanced to Phase 2 only if they demonstrated both functional hearing recovery and structural preservation of the organ of Corti following intratympanic administration. NAC was excluded from subsequent combination experiments because it failed to show significant improvement in ABR thresholds or cochlear morphology despite confirmed perilymphatic penetration. This stepwise selection strategy was implemented to focus mechanistic and combination analyses on agents with demonstrable therapeutic efficacy in the noise-induced hearing loss model. Therapeutic outcomes of combination therapy and DEX monotherapy were systematically compared. ABR threshold measurement was designated as the primary outcome measure. OC morphological analysis and Western blot analysis of inflammatory markers were considered secondary outcomes. A schematic of the experimental design is presented as Figure 1.

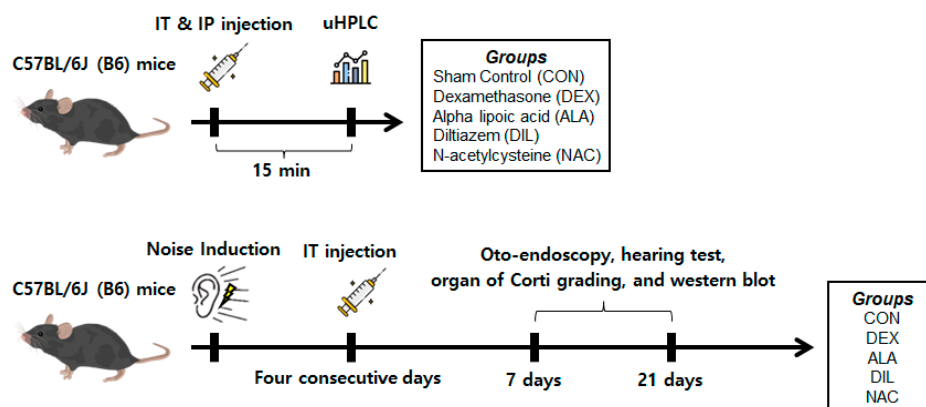
2.4. Drug Administration

In the experiment measuring the perilymphatic concentration of the drug, a single IT injection was administered. In the noise-induced hearing loss model, IT injections were performed daily for four consecutive days to evaluate the therapeutic effect of the drug.

The C57BL/6J mice were sedated using a combination of IP anesthetics (Rompun 0.4 mL/kg and Zoletil 0.6 mL/kg) while positioned on a temperature-regulated surface. IT drug delivery was performed with a 30-gauge needle under a surgical microscope (Zeiss Stativ S2, Jena, Germany). Before drug delivery, a controlled perforation was created in the tympanic membrane separate from the injection site, serving as a ventilation hole to allow

air to escape from the middle ear cavity during IT injection and thereby minimize increased middle ear pressure and drug leakage. The injection volume of 0.05 mL was selected based on established protocols for IT injection in mice, as previously reported, and was designed to ensure complete filling of the middle ear cavity and adequate contact with the round window membrane. The drug solution was injected slowly through a 30-gauge needle to further reduce the risk of excessive middle ear pressure. After injection, all animals were strategically positioned to ensure uniform distribution, maintaining a lateral posture with the treated ear oriented upward for 15 min [14].

(A) Phase 1. Screening of Candidate Drugs



(B) Phase 2. Evaluating Synergetic Effects

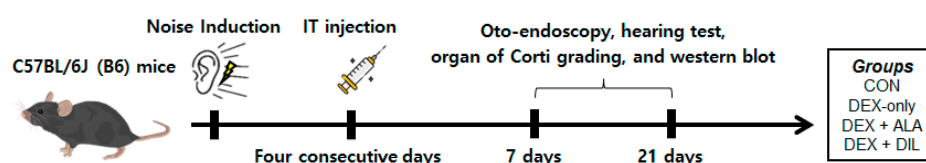


Figure 1. Experimental design schematic. **(A)** Phase 1: Screening of candidate drugs (ALA, DIL, NAC, and DEX) through perilymph concentration analysis (uHPLC), therapeutic efficacy evaluation (ABR, organ of Corti morphology), and mechanistic studies (Western blot). **(B)** Phase 2: Evaluation of synergistic effects by co-administering promising candidates with dexamethasone. IT, intratympanic; IP, intraperitoneal; uHPLC, ultra-high-performance liquid chromatography.

2.5. Oto-Endoscopic Examination

Animals displaying abnormal findings were excluded from the study. Healing progress of the tympanic membrane (TM) was monitored at 1 and 3 weeks after IT injection using a rigid 0° oto-endoscope (2.7 mm outer diameter, 3 cm length, Olympus, Tokyo, Japan) with illumination provided by a Medstar Sharima HGL-100 (Medstar Co., Ltd., Uiwang-si, Gyeonggi-do, Republic of Korea) DC 15 V/150 W halogen light source.

2.6. Perilymph Collection and Dexamethasone Concentration Measurement

In this study, perilymph collection was performed following a methodology established by Salt et al. [12]. Briefly, a retro-auricular incision was made under general anesthesia. The lateral semi-circular canal was exposed and a small perforation was created with a 23-gauge syringe under microscopic view. Perilymph samples were then carefully drawn into capillary tubes (Sigma-Aldrich, St. Louis, MO, USA).

The collected samples underwent comprehensive analysis using an advanced liquid chromatography–tandem mass spectrometry (LC-MS/MS) system. The analytical setup comprised an Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, CA, USA) and a SCIEX QTRAP 6500 LC-MS/MS apparatus (SCIEX, Framingham, MA, USA). Liquid chromatographic separation used an Agilent Eclipse XDB-C18 column

(75 mm × 2.1 mm, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) maintained at 40 °C. The mobile phase was composed of solvent A (0.1% formic acid in deionized water) and solvent B (0.1% formic acid in acetonitrile), which were delivered at a steady flow of 0.4 mL/min. The autosampler, set at 40 °C, used a consistent 5 µL injection volume for all samples. Mass spectrometric analysis used a SCIEX QTRAP 6500 system with a Turbo VTM electrospray ionization source. Data were acquired in positive multiple reaction monitoring mode, with 774 cycles performed in 6 min. Instrumental parameters were carefully optimized, including an ion source potential of 5500 V; a source temperature of 500 °C; and specific voltage settings for declustering, entrance, collision, and cell exit potentials. The system used nitrogen as a curtain gas (35 psi), nebulizer gas (50 psi), and turbo gas (50 psi), ensuring precise and consistent analytical conditions.

2.7. Hearing Test

ABRs were acquired using a Smart EP system (Intelligent Hearing Systems, Miami, FL, USA) equipped with high-frequency transducers (HFT9911-20-0035) and operated with Running High-Frequency software version 2.33. Subdermal needle electrodes were inserted at the vertex (active) and below the left pinna (reference), with signals routed through a preamplifier applying a 0.1–3 kHz bandpass filter. Acoustic stimuli comprised 100-µs clicks presented at 31 Hz and cos²-gated tone bursts at 8, 16, and 32 kHz (1562 µs duration, 21 Hz repetition rate). Stimuli were delivered via insert earphones, with the sound intensity decreasing in 5–10 dB decrements to determine hearing thresholds. Responses were amplified 200,000-fold, bandpass-filtered between 100 and 3000 Hz, and averaged over 1024 sweeps. Two independent evaluators determined the ABR thresholds by identifying the lowest sound intensity at which a reproducible waveform could be clearly observed for each stimulus type.

2.8. OC Morphology Assessment

Following the final auditory evaluation, both experimental and control mice were euthanized under anesthesia, and their cochleae were collected. After careful dissection, the cochleae were perfused through both round and oval windows with a fixative solution containing 2% paraformaldehyde and 2% glutaraldehyde (*v/v*) in a 0.1 M phosphate buffer (pH 7.4) and then post-fixed overnight at 4 °C in the same solution. Tissues were rinsed with 0.1 M phosphate-buffered saline, treated with 1% osmium tetroxide (*w/v*) overnight, and decalcified in 5% EDTA (*w/v*) for two to four days. Following decalcification, specimens were dehydrated using a graded ethanol and propylene oxide series and then embedded in Araldite 502 resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Embedded samples were sectioned into 5 µm slices, stained with toluidine blue dye, and mounted on glass slides for light microscopy.

Morphological assessments were conducted using a Panoramic SCAN II digital scanner (3DHISTECH Ltd., Budapest, Hungary). For each cochlea, six sections were randomly selected from the mid-modiolar plane of the basal, middle, and apical turns. The degree of OC degeneration was evaluated by light microscopy and graded on a scale from 1 to 5 using a modified version of a ranking system proposed by Leake et al. [18], and based on the integrity of supporting cells and overall cytoarchitecture: grade 5 = intact hair cells and normal cytoarchitecture; grade 4 = preserved supporting cells and cytoarchitecture but no hair cells; grade 3 = partial collapse of the OC with identifiable supporting cell types; grade 2 = presence of a low cuboidal cell layer lacking distinguishable supporting cells; grade 1 = complete degeneration with a flattened, undifferentiated epithelial layer. Average grading scores for the basal, middle, and apical regions were compared among the four experimental groups.

2.9. Western Blot Analysis

Protein samples were extracted from cochlear tissues using a lysis buffer (#89900, Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were determined using a bicinchoninic acid assay (#71285, Merck Millipore, Burlington, MA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels and transferred onto polyvinylidene fluoride membranes (#IPVH00010, Merck Millipore, CA, USA). The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline and Tween 20 for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against NF- κ B (#8242, Cell Signaling Technology, Danvers, MA, USA), TNF- α (#11948S, Cell Signaling Technology, MA, USA), IL-6 (#ab290735, Abcam, Cambridge, UK), IL-1 β (#AF-401-NA, R&D System, Minneapolis, MN, USA), and β -actin (#4967, Cell Signaling Technology, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (#7074S, Cell Signaling Technology, MA, USA) and donkey anti-goat (#PA1-28664, Thermo Fisher Scientific, MA, USA) secondary antibodies for 2 h at room temperature. Protein bands were visualized using a West Pico substrate (#34577, Thermo Fisher Scientific, MA, USA), and chemiluminescent signals were detected using a PXi4 imaging system (SynGene, Cambridge, UK) and analyzed in ImageJ (version 1.51, National Institutes of Health, Bethesda, MD, USA).

2.10. Data Analysis and Statistics

All data are presented as group mean $\pm$ standard error of the mean. Statistical analyses were carried out in GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA). Parametric and nonparametric tests were chosen based on the results of the Shapiro–Wilk normality test.

For the uHPLC data, the Mann–Whitney U test was applied. ABR threshold data were analyzed using a Friedman test, followed by Dunn’s post hoc test for repeated measures. Given the small sample size and non-normal distribution of repeated measures, non-parametric Friedman testing was selected. Between-group comparisons at each time point were performed using the Kruskal–Wallis test with Dunn’s multiple comparisons post hoc test. Morphological findings of the OC by light microscopy and the results of Western blot analyses were also analyzed using the Kruskal–Wallis test, followed by Dunn’s post hoc test.

A *p*-value less than 0.05 was considered statistically significant. In all figures, significance is indicated as follows: * *p* < 0.05, ** *p* < 0.01, and *** *p* < 0.001.

3. Results

3.1. Phase 1—Screening of Candidate Drugs

3.1.1. Oto-Endoscopic Findings of the TM

One week after IT injection, inflammatory reactions were observed in the ALA group; complete recovery of all tympanic membranes was achieved by week 3 (Figure 2A).

3.1.2. Perilymph Concentration of Injected Drugs

Perilymph samples obtained 15 min after a single IT injection were characterized by higher drug concentrations in all groups compared with samples associated with IP administration. Among these, DEX and ALA showed significant increases in perilymph concentrations following IT injection compared with IP delivery (IP-DEX 31.63 ± 7.15 ppb vs. IT-DEX 2064.28 ± 828.05 ppb, *p* = 0.001; IP-ALA 6.89 ± 1.54 ppb vs. IT-ALA 5409.39 ± 1339.16 ppb, *p* = 0.002; IP-DIL 28.46 ± 7.80 ppb vs. IT-DIL 95.97 ± 37.15 ppb, *p* = 0.178; IP-NAC 5.65 ± 1.77 ppb vs. IT-NAC 168.16 ± 33.38 ppb, *p* = 0.004) (Figure 2B).

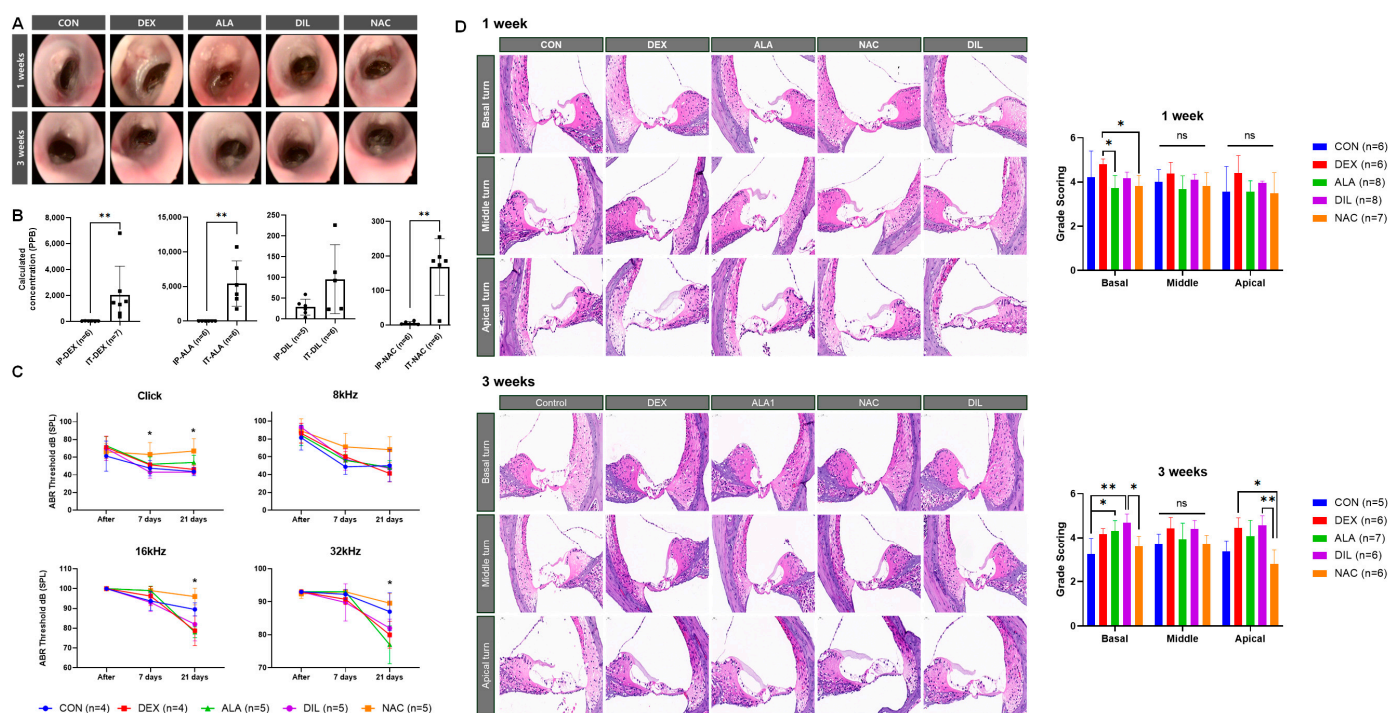


Figure 2. (A) Oto-endoscopic findings 1 and 3 weeks after IT injections. Oto-endoscopic examination revealed similar-sized TM perforations with no otorrhea. (B) uHPLC results 15 min after injection. All candidate drugs successfully diffused into the perilymph. However, significant differences were confirmed only for DEX, ALA, and NAC groups ($p < 0.05$). (C) Significant improvements in ABR thresholds were observed for DIL (click stimuli, days 7 and 21), DEX and ALA (16 kHz, day 21), and ALA (32 kHz, day 21) compared with NAC treatment ($p < 0.05$). (D) Results of OC grading scores. NAC demonstrated no significant impact on OC morphology, whereas ALA and DIL exhibited significant recovery 3 weeks after IT injection. ns, non-specific; * $p < 0.05$; ** $p < 0.01$.

3.1.3. ABR Results

Hearing recovery was assessed at 1 and 3 weeks following exposure to noise in the mice with noise-induced hearing loss. Among the four tested agents, NAC had no therapeutic effect on ABR thresholds, while DEX, ALA, and DIL demonstrated significant improvements in ABR thresholds. However, repeated measures analysis using the Friedman test revealed no significant differences in hearing recovery among treatment groups over the entire study period ($p > 0.05$).

To examine potential differences at individual time points, Kruskal–Wallis tests compared group means at each assessment. For click-evoked ABRs, the DIL group showed significantly improved thresholds compared with the NAC group at both 7 days (DIL: 57.00 ± 5.39 dB vs. NAC: 71.00 ± 6.78 dB; $p = 0.032$) and 21 days (DIL: 43.00 ± 1.23 dB vs. NAC: 67.00 ± 6.25 dB; $p = 0.014$). At the 16 kHz tone-burst stimulus on day 21, both the DEX (78.75 ± 3.75 dB; $p = 0.037$) and ALA (78.00 ± 1.23 dB; $p = 0.010$) groups demonstrated significantly lower ABR thresholds compared with the NAC group (96.00 ± 1.87 dB). Similarly, for the 32 kHz stimulus on day 21, significantly lower thresholds were evident in the ALA group (77.00 ± 2.55 dB) compared with the NAC group (89.60 ± 1.29 dB; $p = 0.016$) (Figure 2C).

3.1.4. Organ of Corti Morphology

Following noise exposure and four consecutive days of IT injection with respective drugs, at 1 week post-treatment, the DEX group showed significantly superior OC morphology (4.22 ± 0.48) in the basal turn compared with the ALA (3.73 ± 0.20 , $p = 0.014$) and

NAC (3.81 ± 0.18 , $p = 0.023$) groups, while no significant differences were observed in the middle and apical turns ($p > 0.05$).

At 3 weeks post-treatment in the basal turn, the ALA (4.31 ± 0.18 , $p = 0.038$) and DIL (4.68 ± 0.16 , $p = 0.002$) groups showed higher OC grade scores compared with the control group (3.22 ± 0.34), and the DIL group achieved a higher score compared with the NAC group (3.58 ± 0.20 , $p = 0.019$). No significant differences were observed in the middle turn; in the apical turn, the NAC group (2.78 ± 0.26) showed significantly lower OC grade scores compared with the DEX (4.45 ± 0.19 , $p = 0.011$) and DIL (4.58 ± 0.17 , $p = 0.005$) groups. Representative OC morphology and analysis results are presented in Figure 2D.

3.1.5. Western Blot Analysis of Inflammatory Markers

IT-NAC was excluded from further analysis due to its lack of efficacy in inner ear recovery, and Western blot analysis was performed to compare the mechanisms of action between ALA and DIL. At 1 week after noise exposure, expression of TNF- α proteins was significantly reduced in the DEX (0.77 ± 0.08 , $p = 0.015$) and ALA (0.68 ± 0.09 , $p = 0.002$) groups compared with the control group (1.18 ± 0.12). In addition, NF- κ B expression was significantly suppressed in the ALA (0.67 ± 0.07 , $p = 0.001$) and DIL (0.82 ± 0.06 , $p = 0.026$) groups compared with the control group (1.16 ± 0.09). No significant differences were observed for IL-1 β among groups. IL-6 expression was significantly suppressed in the ALA (0.65 ± 0.05 , $p < 0.001$) and DIL (0.9 ± 0.10 , $p = 0.003$) groups compared with the control group (1.47 ± 0.17), with the ALA group demonstrating significantly greater suppression than the DEX group (1.09 ± 0.0 , $p = 0.023$).

At 3 weeks post-treatment, TNF- α protein expression was significantly reduced in the DIL group (0.83 ± 0.12 , $p = 0.010$) compared with the control group (1.38 ± 0.08), and NF- κ B expression was significantly decreased in the DIL group (0.47 ± 0.08 , $p = 0.002$) compared with the control group (0.79 ± 0.04). IL-1 β expression was significantly higher in the control group compared with all other groups ($p < 0.05$), with no significant differences observed among the treatment groups. No significant differences in IL-6 expression were observed among groups. (Figure 3).

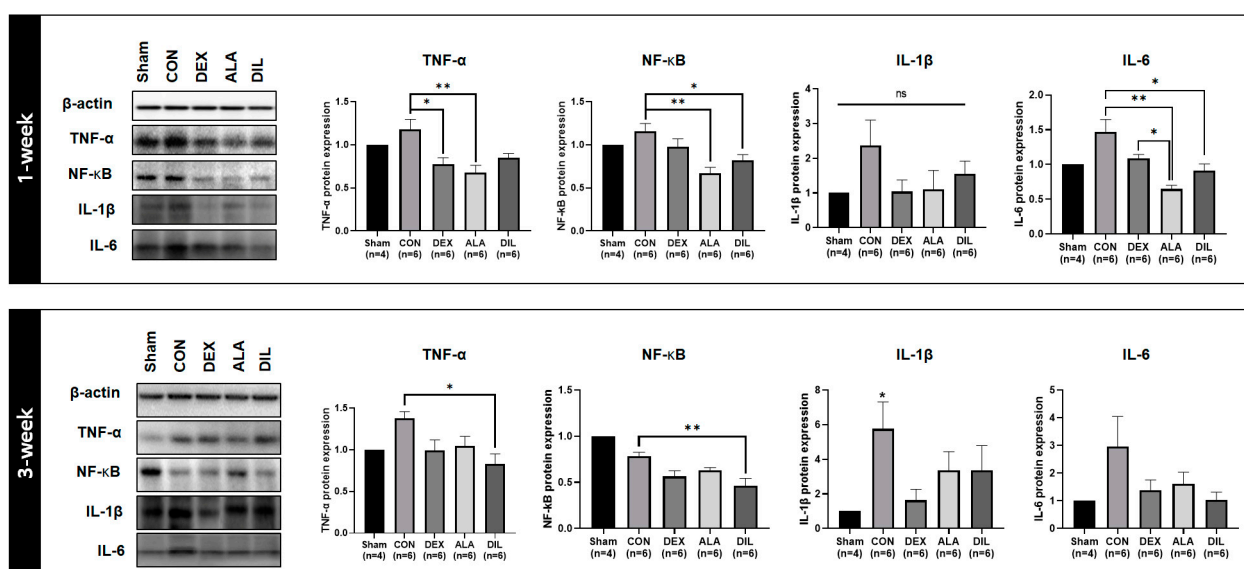


Figure 3. Western blot analysis revealed that DEX, ALA, and DIL all reduced NF- κ B and TNF- α expression at week 1. Among these treatments, DIL exhibited the strongest suppressive effect on both inflammatory markers by week 3. ns, non-specific; * $p < 0.05$; ** $p < 0.01$.

3.2. Phase 2—Evaluating Synergistic Effects

3.2.1. Oto-Endoscopic Findings of the TM

Oto-endoscopic examination revealed severe inflammation in the middle ear and TM in all mixed-treatment groups (Figure 4A).

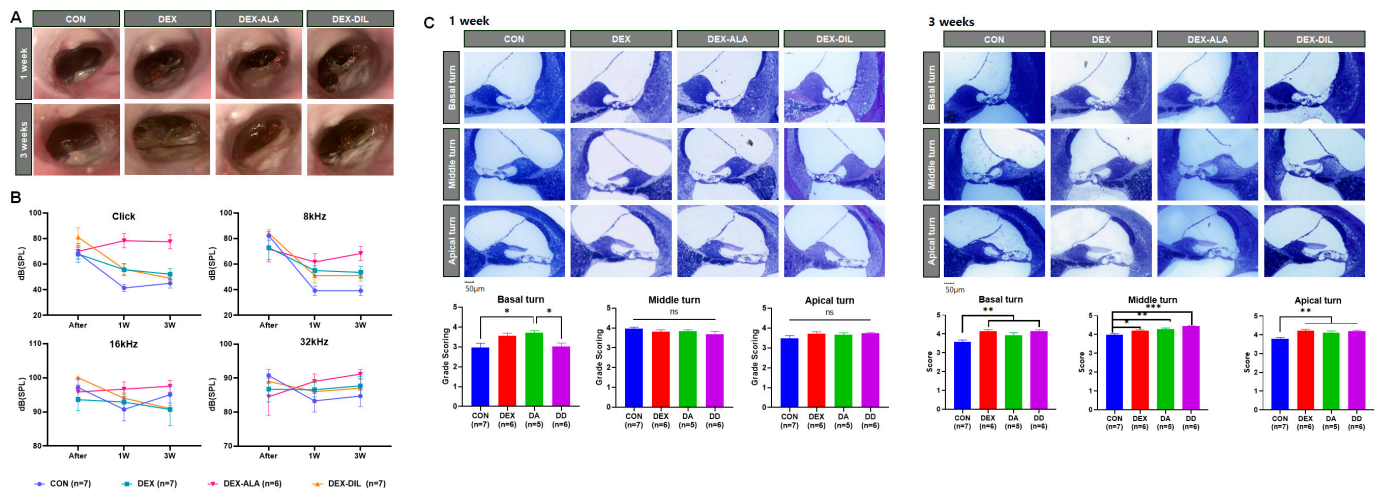


Figure 4. (A) Oto-endoscopic findings 1 and 3 weeks after IT injections. Both DEX-ALA and DEX-DIL groups showed larger perforation and inflammation of the TM compared with the control and DEX groups. (B) Neither the DEX-ALA nor the DEX-DIL group showed a significant improvement in ABR thresholds compared with DEX alone. (C) Results of OC grading scores: At week 3, the DEX only, DEX-ALA, and DEX-DIL groups all showed significantly superior scores compared with the control group; however, no significant differences were observed among these three treatment groups. ns, non-specific; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

3.2.2. ABR Results

No significant differences in ABR results were evident among the groups (Figure 4B).

3.2.3. Organ of Corti Morphology

At 1-week post-treatment, the DEX-ALA group showed significantly superior OC grade scores (3.7 ± 0.11) in the basal turn compared with the control (2.96 ± 0.22 , $p = 0.015$) and DEX-DIL (3.03 ± 0.17 , $p = 0.030$) groups, while no significant differences were observed in the middle and apical turns.

At 3 weeks post-treatment, in the basal turn, the DEX-only (4.14 ± 0.09 , $p = 0.003$) and DEX-DIL (4.13 ± 0.09 , $p = 0.003$) groups showed significantly superior OC grade scores compared with the control group (3.57 ± 0.11). In the middle and apical turns, all treatment groups demonstrated significantly superior OC grade scores compared with the control group (middle turn 3.99 ± 0.06 , apical turn 3.80 ± 0.06): DEX (middle turn 4.20 ± 0.05 , $p = 0.034$; apical turn 4.22 ± 0.08 , $p = 0.001$), DEX-ALA (middle turn 4.29 ± 0.06 , $p = 0.004$; apical turn 4.12 ± 0.08 , $p = 0.018$), and DEX-DIL (middle turn 4.44 ± 0.04 , $p < 0.001$; apical turn 4.20 ± 0.05 , $p = 0.002$).

Representative images and analysis results are presented in Figure 4C.

3.2.4. Western Blot Analysis of Inflammatory Markers

Western blot analysis at 1-week post-treatment showed no drug-induced reduction in inflammatory markers. TNF- α was significantly increased in the DEX-ALA group (1.69 ± 0.20) compared with the control group (1.00 ± 0.12 , $p = 0.045$), and NF- κ B was also significantly increased in the DEX-only (2.11 ± 0.32 , $p = 0.017$) and DEX-DIL (2.06 ± 0.28 , $p = 0.023$) groups compared with the control group (1.00 ± 0.12).

Western blot analysis at 3 weeks post-treatment revealed no significant differences in TNF- α , NF- κ B, or IL-1 β expression among groups. However, IL-6 expression was significantly decreased in the DEX-ALA group (0.26 ± 0.09) compared with the control group (1.00 ± 0.19 , $p = 0.030$) (Figure 5).

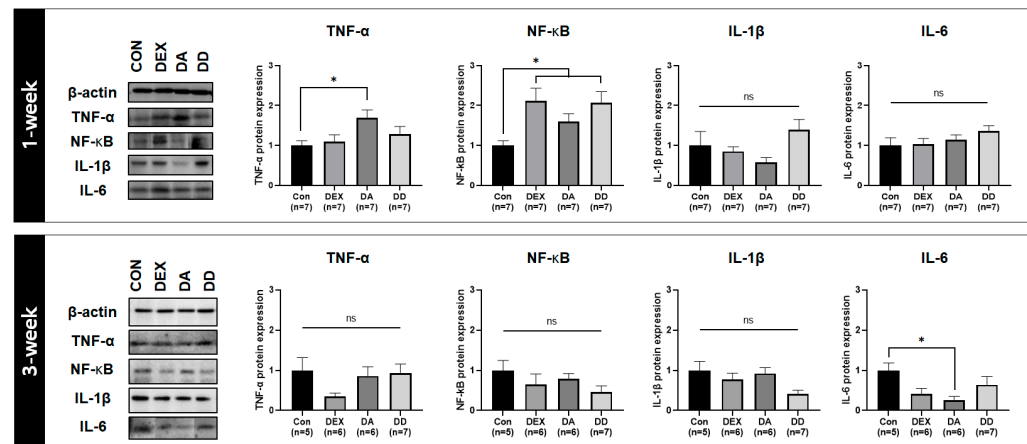


Figure 5. Western blot analysis showed elevated TNF- α and NF- κ B levels at week 1. By week 3, the IL-6 level was reduced in the DEX-ALA group; however, no significant differences were observed among groups for the other three inflammatory markers. ns, non-specific; * $p < 0.05$.

4. Discussion

This study systematically evaluated the therapeutic potential of three clinically available agents—ALA, DIL, and NAC—to improve the effect of IT-DEX using a noise-induced hearing loss murine model. Among the three agents, IT-ALA and IT-DIL had significant effects on both ABR thresholds and OC morphology that were comparable to those of IT-DEX. However, IT-NAC failed to demonstrate a meaningful therapeutic effect. Although co-administration of ALA or DIL with DEX resulted in comparable or modestly enhanced preservation of cochlear morphology, no additive improvement in auditory function was observed. Moreover, increased TM inflammation in the combination groups may have attenuated any potential synergistic effect.

IT-DEX remains the most widely recognized and clinically effective treatment for acute disorders of the inner ear [19]. Nevertheless, its therapeutic efficacy is often limited, creating the need for adjunctive agents capable of producing synergistic effects. Selection of candidate drugs for combination therapy requires several prerequisites. First, the drug must be suitable for IT delivery, which necessitates minimal adverse effects on the middle ear and a sufficiently low molecular weight to enable passage through the round window membrane [20]. Previous studies have demonstrated that ALA, DIL, and NAC fulfill these conditions, and our present findings using oto-endoscopic assessment and uHPLC further confirmed their safety and permeability.

Second, candidate agents should act via mechanisms distinct from those associated with DEX, providing complementary therapeutic effects. DEX exerts its efficacy primarily as an anti-inflammatory agent, whereas ALA and NAC are characterized as antioxidants [21–26]. DIL, traditionally evaluated in the clinical setting as a systemic calcium channel blocker for vasodilatory therapy in ISSNHL [15], has more recently been shown to exert protective effects by preventing the influx of calcium into outer hair cells, reducing lipid peroxidation and limiting mitochondrial permeability [27,28]. Moreover, a growing body of cardiovascular literature indicates that calcium channel blockers, including diltiazem, also possess intrinsic antioxidant properties [29]. These findings suggest that DIL can facilitate cochlear protection through mechanisms independent of DEX.

The Western blot analyses revealed distinct expression patterns of inflammatory markers in the ALA and DIL groups compared with DEX. Notably, both ALA and DIL suppressed NF- κ B more strongly than did DEX, likely because of their antioxidant-driven mechanisms of action, in contrast to the glucocorticoid-mediated anti-inflammatory pathway of DEX [30]. Consistent with this difference, IL-6 expression, which is largely regulated at the transcriptional level by NF- κ B, was more effectively reduced in the ALA and DIL groups than in the DEX group. In contrast, IL-1 β showed minimal additional suppression, which is mechanistically plausible because pro-IL-1 β requires NLRP3 inflammasome- and caspase-1-dependent processing to become biologically active. Therefore, treatments primarily targeting oxidative stress and NF- κ B signaling may exert limited influence on IL-1 β maturation compared with their effects on IL-6 [31].

Consistent with these expectations, NAC failed to produce a meaningful therapeutic benefit as a monotherapy in our study and was excluded from further consideration. Although NAC has demonstrated protective effects against oxidative stress in several experimental and clinical studies [11,13,17,21,23,25], its efficacy is highly dependent on both timing and sustained cochlear exposure. Despite having a relatively low molecular weight (163.2 Da) that is theoretically favorable for round window membrane permeation, NAC possesses high aqueous solubility and undergoes rapid clearance from the middle ear cavity, which may result in insufficient duration of cochlear exposure to achieve therapeutic concentrations. Furthermore, the single daily IT dosing protocol employed in this study may not have maintained sustained perilymph concentrations of NAC necessary for effective antioxidant activity. Potential strategies to enhance NAC bioavailability in future studies include sustained-release formulations such as thermosensitive hydrogels, nanoparticle encapsulation to prolong middle ear retention, or more frequent dosing protocols.

Finally, an effective adjunct should not only prove beneficial as a monotherapy but also enhance outcomes when combined with DEX. While both ALA and DIL demonstrated efficacy individually, neither produced clear synergistic effects in combination with DEX. Although OC morphology was modestly improved compared with DEX monotherapy, these changes were not significant, and ABR measurements revealed no additional functional recovery. In fact, the DEX + ALA group demonstrated the poorest auditory outcome among the treatment arms. This disconnect between structural preservation and functional outcome may be attributable to several factors: the morphological grading system evaluates gross cytoarchitectural integrity but may not detect subtle subcellular damage such as synaptic ribbon loss or stereocilia disruption; and more importantly, the severe middle ear inflammation observed in all combination groups may have introduced a conductive hearing component that masked any underlying sensorineural improvement [32,33]. Thus, the lack of synergy observed in this study likely reflects limitations related to local tissue tolerability and IT drug delivery rather than true pharmacodynamic antagonism between the agents.

Several factors may explain these unexpected results. First, co-administration appeared to exacerbate middle ear inflammation, as confirmed by oto-endoscopic evaluation, which likely counteracted potential therapeutic benefits. Second, although DEX targets inflammation and ALA and DIL primarily target oxidative stress, these two pathways are closely interlinked. Inflammatory processes generate reactive oxygen species (ROS), which exacerbate inflammation, establishing a self-perpetuating cycle [34]. Even if different drugs interrupt distinct components of this cycle, the ultimate outcome may converge toward a similar endpoint, such that combining agents does not provide an additive benefit. In this regard, although ALA and DIL act through antioxidant pathways, the final effect of attenuating the vicious cycle of ROS and inflammation may overlap with that achieved by DEX, limiting the potential for true synergy.

The findings of this study may have important implications for future investigation of clinical management of acute inner ear disease. While IT-DEX remains the gold standard treatment [9], our results demonstrate that ALA and DIL individually exhibit therapeutic effects comparable to those of DEX, mediated through distinct antioxidant pathways, suggesting their potential as candidate agents worthy of further clinical investigation. Moreover, the use of repurposed agents with established clinical safety profiles could accelerate translational application compared with investigational drugs. This study also shows that combination therapy does not necessarily translate into enhanced therapeutic efficacy, and that drug-induced middle ear inflammation may negate any potential benefits. These findings underscore the need to carefully evaluate not only pharmacodynamic synergy but also local tissue tolerability when designing IT drug regimens for clinical use.

Several limitations of this study should be acknowledged. First, the experiments were conducted in a murine model, which may not fully recapitulate the anatomical and immunological characteristics of the human middle and inner ear. The murine middle ear cavity is considerably smaller than that of humans, resulting in proportionally greater filling by the 0.05 mL injection volume and potentially amplifying local inflammatory responses. IT injection in mice is also more likely to damage the tympanic membrane compared with an injection in humans, raising the possibility that TM and middle ear inflammation may have been overestimated in our model. Furthermore, species-specific differences in immune cell composition, cytokine signaling dynamics, and round window membrane permeability may influence both drug distribution and therapeutic response, limiting the direct applicability of these findings to human patients [35].

Second, the findings are restricted to a noise-induced hearing loss model. Although IT-DEX is the most widely used therapy for acute diseases of the inner ear such as ISSNHL, no established animal model faithfully reproduces such pathology [35]. ISSNHL is considered a heterogeneous condition involving diverse etiologies, including viral infection, vascular compromise, autoimmune reactions, and membrane rupture, each of which may involve distinct inflammatory and oxidative stress dynamics that differ fundamentally from the uniform oxidative stress-driven damage produced by noise exposure. While acute hearing loss in animals is typically induced using exposure to noise or ototoxic drugs, the therapeutic effects in clinical cases of acute inner ear disease may differ substantially. Therefore, our findings should be interpreted as preclinical proof-of-concept rather than direct clinical evidence, and future studies in models that more closely approximate human inner ear pathology are warranted.

Third, despite their individual efficacy, both ALA and DIL ultimately failed to demonstrate synergistic effects when combined with DEX. The severe middle ear inflammation in the combination groups represents a major confounding factor that precludes definitive conclusions regarding pharmacodynamic synergy or antagonism between these agents.

Future research should aim to develop vesicular systems that enable dual-drug delivery while minimizing local inflammation, as this represents a promising therapeutic direction. Several strategies merit consideration. First, microcrystalline formulations represent a promising approach; a recent study demonstrated that IT administration of DEX and ALA in microcrystalline form was safe for the middle ear, although the synergistic effect was confirmed only *in vitro* using a cisplatin-induced ototoxicity model, limiting its translational relevance [36]. Second, vesicle-based approaches such as liposomal or polymeric nanoparticles could simultaneously deliver multiple agents while providing a protective barrier that reduces direct contact between the drug formulation and middle ear mucosa, potentially attenuating inflammatory responses [37,38]. Third, dose-reduction strategies or sequential administration protocols—in which individual agents are delivered at separate time points rather than simultaneously—may help mitigate the cumulative

local irritation observed with co-administration. Fourth, the use of biocompatible hydrogel carriers that provide sustained drug release could reduce the need for repeated injections and thereby minimize mechanical trauma to the tympanic membrane. Additionally, although studies of candidate agents have focused on anti-inflammatory or antioxidant effects, these two mechanisms overlap substantially, which may limit the likelihood of achieving true synergy [34]. Identifying drugs that target distinct biological pathways beyond inflammation and oxidative stress—such as apoptotic cascades, mitochondrial biogenesis, or neurotrophic signaling—may be necessary to realize meaningful synergistic effects in combination IT therapy.

5. Conclusions

This study found that IT administration of DEX, ALA, and DIL provided protective effects on hearing and cochlear morphology, whereas NAC was ineffective. ALA and DIL exhibited distinct anti-inflammatory and antioxidant mechanisms compared with DEX, suggesting their potential as alternative therapeutic agents. However, neither produced synergistic effects when combined with DEX, and combination therapy induced greater middle ear inflammation, which likely offset the potential benefits. While ALA and DIL may be promising candidates for IT therapy, their concurrent use with DEX does not enhance efficacy. Future research should focus on safer dual-drug delivery systems and exploration of agents targeting pathways beyond inflammation and oxidative stress to achieve meaningful synergy.

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Abbreviations

The following abbreviations are used in this manuscript:

ALA	alpha-lipoic acid
DIL	diltiazem
NAC	N-acetylcysteine
IT	intratympanic
DEX	dexamethasone
ISSNHL	idiopathic sudden sensorineural hearing loss
ABR	auditory brainstem response

OC	organ of Corti
IP	intraperitoneal
uHPLC	ultra-high-performance liquid chromatography
NF- κ B	nuclear factor kappa-B
TNF- α	tumor necrosis factor alpha
IL	interleukin
TM	tympenic membrane
LC-MS/MS	advanced liquid chromatography–tandem mass spectrometry
ROS	reactive oxygen species

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