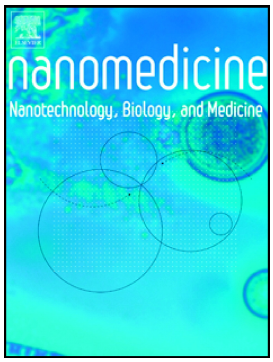


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# Intratympanic administration of alpha-lipoic acid-loaded Pluronic F-127 nanoparticles ameliorates acute hearing loss

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*Abbreviations: NP*, nanoparticle; *ALA*, alpha-lipoic acid; *ROS*, reactive oxygen species; *RWM*, round window membrane; *PEG*, polyethylene glycol; *NR*, Nile red.

Conflicts: No author has any possible conflict of interest.

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**Abstract**

We used antioxidant-containing nanoparticles (NPs) to treat acute hearing loss. Alpha-lipoic acid (ALA) served as the antioxidant; we employed Pluronic F127 to fabricate NPs. *In vitro*, ALA-NPs protected cells of the organ of Corti in HEI-OC1 mice, triggering nuclear translocation of NRF2 and increases in the levels of antioxidant proteins, including Nrf2, HO-1, SOD-1, and SOD-2. *In vivo*, the hearing of mice that received ALA-NP injections into the middle ear cavity was better preserved after induction of ototoxicity than in control animals. The cochlear Nrf2 level increased in test mice, indicating that the ALA-NPs protected hearing via the antioxidant mechanism observed *in vitro*. ALA-NPs effectively protected against acute hearing loss by activating the Nrf2/HO-1 pathway.

**Keywords:** alpha-lipoic acid; nanoparticle; intratympanic injection; hearing loss; antioxidant; ototoxicity.

## Background

Reactive oxygen species (ROS) are chemically reactive molecules produced by the partial reduction of oxygen. They are formed naturally by normal metabolism of oxygen, and play important roles in cell signaling and homeostasis.[1] However, production of excess ROS can overwhelm the body's antioxidant defenses; this is known as oxidative stress. Tissues respond to mild oxidative stress by increasing antioxidant production, but severe oxidative stress can cause cell injury and death.[2] Several types of sensorineural hearing loss are associated with increased ROS production. In addition to noise-induced and ototoxic hearing loss,[3,4] ROS are also associated with sudden or senile hearing loss.[5,6] Recent trials have indicated that antioxidants show promise for preventing cisplatin-induced hearing loss in pediatric patients with cancer.[7,8]

Alpha-lipoic acid (ALA) is an antioxidant that reportedly protects against ototoxicity *in vitro* and *in vivo*.[9-12] Also, systemic pre- or post-treatment with ALA was effective in an animal model of cisplatin-induced hearing loss.[11] However, systemic administration of ALA has several drawbacks. Following systemic administration, the cochlea absorbs only a small fraction of the drug, due to the blood–labyrinthine barrier. Thus, high drug levels are required for therapeutic efficacy, which may be associated with severe side-effects. Also, use of drugs to prevent the hearing loss caused by anticancer agents may reduce the efficacy of the latter.[13] As an alternative, free drug can be injected into the middle-ear cavity, but this can result in an inadequate concentration of the drug beyond the round-window membrane (RWM).[14] Furthermore, lipophilic drugs are dissolved in an organic solvent such as dimethyl sulfoxide (DMSO) for intratympanic injection, but such solvents may be ototoxic.[15]

We attempt to overcome these issues by injecting ALA-loaded nanoparticles (ALA-NPs) into the middle-ear cavity. Intratympanic injection of steroid-containing NP to the inner ear showed therapeutic efficacy for acute hearing loss.[16-18] To our knowledge, this is the first report of intratympanic injection of ALA contained in NPs to reduce ROS levels and ameliorate hearing loss,

which represents a novel therapeutic modality.

To date, various approaches have been used to promote the passage of drugs through the RWM. These include micro-perforating the RWM using a microneedle, drilling a hole in the cochlea, and guiding nanoparticles into the cochlea using a magnetic field.[17,19-21] However, use of these methods in clinical practice is problematic; they are either too complicated or too invasive. We reported previously that coating of NP with polyethylene glycol (PEG) increases RWM permeability.[22] Therefore, here, we used Pluronic F-127 as the shell component of NP because it contains two PEG groups and is approved by the United States Food and Drug Administration for intravenous infusion (Fig. 1).

Herein, we investigated the antioxidant activity of ALA-NP *in vitro* and evaluated their therapeutic effect on hearing loss *in vivo* using a mouse model. The antioxidant activity of ALA is mediated by quenching of free radicals, chelation of metals, and recycling of antioxidants.[23] It was recently shown that the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) antioxidant pathway is involved in the antioxidant activity of ALA; we thus explored whether ALA-NPs affected this pathway.[24]



## Methods

### *Preparation of nanoparticles*

Pluronic F-127, (±)-alpha-lipoic acid (ALA), Nile red, and flax seed oil were obtained from Sigma-Aldrich (St. Louis, MO). DMSO was purchased from Samchun (Seoul, Gangnam-gu, Republic of Korea). Ethanol was purchased from Duksan (Seongnam, Gyeonggi-do, Republic of Korea). Glycerin was obtained from Junsei Chemical Co., Ltd. (Nihonbashi-honcho, Chuo-ku, Tokyo, Japan).

ALA-NPs were fabricated by an oil-in-water emulsion method. We dissolved 40 mg of Pluronic

F-127 in 2.0 mL of a 2% (v/v) glycerol/water solution. ALA (20 mg) and flax seed oil (100 mg) were dissolved in 700 μL of ethanol and the mixture was slowly added to the Pluronic F-127 solution. The mixture was dispersed by sonication using a C505 probe sonicator (Sonics & Materials Inc., Newtown, CT) for 20 min. Unloaded ALA was removed by dialysis against distilled water for 1 h using a 25 kDa molecular weight cutoff membrane.

The size of the ALA-NPs was measured at 25°C in phosphate-buffered saline (PBS; pH 7.4) using a Zetasizer (Nano ZS90; Malvern Instruments, Malvern, UK). The morphology of the ALA-NPs was visualized by transmission electron microscopy (TEM) with negative staining using 2% (w/v) uranyl acetate. The encapsulation efficiency (EE) of the ALA-NPs was determined based on the absorbance at 365 nm of ALA using a Synergy H1 Hybrid Multi-Mode Reader (Biotek Instruments, Inc., Winooski, VT), and was calculated as follows:

EE (%) = Amount of ALA in nanoparticles ÷ total amount of ALA added × 100%

ALA was dissolved in a reagent solution (3 mL of Britton–Robinson buffer [pH 2.2], 2 M potassium chloride, and 0.01 M palladium(II) chloride), which enhances its absorbance of light.[25] To evaluate the release of ALA, ALA-NPs were dialyzed against PBS (pH 7.4). Aliquots of the external solution were removed at predetermined time points and subjected to assay of the ALA concentration.

*In vitro antioxidant effect of the ALA-NPs*

HEI-OC1 mouse organ of Corti cells were used as an *in vitro* model.[26] HEI-OC1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 U/mL recombinant mouse interferon-γ in a humidified 10% $CO_2$ environment at 33°C. Free ALA reportedly exerts an antioxidant effect via the NRF2/HO1 antioxidant pathway.[24] To determine whether ALA-NPs also exert an NRF2/HO1-dependent antioxidant effect, HEI-OC1 cells were transfected with an siRNA for *NRF2* (Invitrogen, Carlsbad, CA) using Lipofectamine RNAiMax (Invitrogen) for 24 h, and the levels of antioxidant proteins were assayed.

We determined the maximum safe dosage of ALA-NP by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol (EZ-Cytox; Daeil Lab, Seoul, Republic of Korea). Also, to assess the protective effect against ototoxicity, the cells were treated with 5 mM kanamycin in the presence or absence of ALA-NP. The optical density at 450 nm was determined in triplicate using a microplate reader (Bio-Rad Laboratories, Hercules, CA) and normalized to the value of the control.

To assess the effect of ALA and ALA-NPs on the nuclear translocation of Nrf2, the nuclear fraction was extracted from HEI-OC1 cells using RSB solution (20 mM Tris-HCl [pH 7.4], 10 mM NaCl, and 2 mM $MgCl_2$). After centrifugation at 600 × g for 5 min, 10 volumes of RSB solution were added, and the cells were incubated for 12 min on ice. The cells were homogenized 12 times using a Dounce homogenizer, and the nuclear extract was generated by centrifugation at 300 × g for 3 min. Western blotting was performed using an antibody against Nrf2 (Abcam, Cambridge, UK) as described below, and the nuclear and cytosolic fractions were confirmed by detecting histone H3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively.

HEI-OC1 cells were treated with free ALA or ALA-NPs in the presence or absence of kanamycin for 24 h. Next, the cells were harvested using radioimmunoprecipitation assay buffer (20 mM Tris–HCl [pH 7.4], 0.01 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 mM $Na_3VO_4$) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Equal amounts of lysates were subjected to Western blotting using primary antibodies against Nrf2, superoxide dismutase 1 (SOD1) (Abcam), HO1, actin, GAPDH (Cell Signaling Technology, Danvers, MA), and SOD2 (Novus Biologicals, Littleton, CO).

2', 7'-Dichlorodihydrofluorescein (DCF-DA) is a chemically reduced form of fluorescein that is used as a marker of ROS. HEI-OC1 cells were treated with free ALA or ALA-NPs in the presence or absence of kanamycin, and fluorescence signals were visualized using H2DCFDA (Thermo Fisher

Scientific, Waltham, CA) according to the manufacturer's protocol. The green fluorescence from the cells was confirmed by fluorescence microscopy (Eclipse TE300, Nikon, Japan) and by fluorescence-activated cell sorting (FACS Canto™, Becton Dickinson and Co., Franklin Lakes, NJ).

*In vitro permeation tests using an artificial mucosal membrane*

*In vitro* permeation of the ALA-NPs was evaluated using an artificial mucosa created from human, oral mucosal cells (Neoderm-OD; Tego Science, Seoul, South Korea). The membrane was transferred to 12-well plates filled with cell culture medium. Free ALA (1 mg/mL) or approximately 10 mg/mL of ALA-NPs containing 1 mg/mL of ALA was placed on top of the artificial mucosa. After 1 and 24 h, the culture media under the mucosal membrane was collected and drug concentrations measured via UHPLC/tandem mass spectrometry (1290 Infinity II/Qtrap 6500; Sciex, Washington DC, USA).[27]

*In vivo applications*

Eight-week-old male C57/BL6 mice (Daehan Science, Daejeon, ROK; weight 20–23 g) were used in the *in vivo* experiments to examine the absorption of NP in the cochlea and their effect on hearing preservation.[28,29] NPs were injected into the middle ear using a surgical method described previously.[30] Before surgery, the mice were anesthetized using a mixture of 30 mg/kg Zoletil (Virbac, Carros, France) and 10 mg/kg Rompun (Bayer, Leverkusen, Germany), placed on a thermoregulated heat pad in the supine position, a midline incision was made, and the left-side bulla was exposed. A bullar hole was created using fine forceps, and an NP solution (5.0 mg/mL) was injected into the bulla cavity using an insulin syringe. The bullar hole was closed using surrounding muscle tissue to prevent leakage of the solution, and the operation was completed after suturing. To reflect clinical practice, we did not use gel foam to retain the drug in the middle ear. Next, Rimadyl (1.0 mg/kg; Pfizer, Walton Oaks, UK) was injected to relieve pain. Baytril (10 mg/kg; Orion, Hamburg,

Germany) was intraperitoneally injected once daily as prophylaxis against middle-ear infection.

To examine the absorption of NP in the cochlea, nanoparticles containing Nile red (NR-NP) were injected into the middle-ear cavity of normal mice as described above. The cochlea was sampled 24 h later to confirm the absorption of NP, rinsed with tap water for 1 min to remove outer surface particles, immersed in 4% (v/v) paraformaldehyde (Merck, Darmstadt, Germany) for 20 min, rinsed with PBS, and the area of the auditory epithelium and the spiral limbus of the middle turn were dissected for preparation of whole-mounts. After mounting using Vectashield Mounting Medium, the tissues were stained with DAPI (Vector Laboratories, Burlingame, CA), and NP uptake was observed under a confocal microscope (LSM5 Live Configuration Variotwo VRGB; Zeiss, Jena, Germany).

To assess the effect of the ALA-NPs on hearing preservation, we injected the mice with saline, free ALA, or ALA-NPs. Ototoxicity was induced by subcutaneous injection of kanamycin (500 mg/kg; Sigma-Aldrich, St. Louis, MO) followed 30 min later by intraperitoneal injection of furosemide (120 mg/kg; Sigma-Aldrich).[22] Hearing was tested by assessing the auditory brainstem response (ABR) at 4 and 7 days after surgery.

An ABR was evoked 4 and 7 days after induction of ototoxicity using a System III Evoked Potential Workstation (Tucker-Davis Technologies, Alachua, FL). Briefly, mice were anesthetized using a mixture of 30 mg/kg Zoletil (Virbac, Carros, France) and 10 mg/kg Rompun (Bayer, Leverkusen, Germany) and placed on a heating pad inside a soundproof acoustic chamber. For ABR testing, click and tone-burst stimuli (4, 8, 16, and 32 kHz) were presented by an MF-1 magnetic speaker (Tucker-Davis Technologies, Alachua, FL) from 90 to 20 dB sound pressure level (SPL) in 5–10 dB SPL steps.[31] The click stimuli were 0.1 ms in duration and the tone-burst stimuli were 5 ms in duration (2.5 ms each for rise and decay, thus without a plateau). Threshold responses were defined as the sound pressure levels at which the peak amplitudes of the evoked responses (latency, 2.5–7.5 ms) were greater than two standard deviations above the average background activity.

The cochleae were harvested 40 h after surgery and subjected to Western blotting to determine the

levels of antioxidant proteins. The cochleae were homogenized using TissueLyser II (Qiagen) and processed as were HEI-OC1 cells.

After measurement of the ABR at 7 days, the mice were euthanized and the cochleae were collected, fixed for 24 h, and decalcified for 2 weeks as described above. Using a sharp-angled micro-scalpel, the bony and membranous labyrinths and the tectorial membrane were carefully removed to expose the organ of Corti, which was sectioned into two portions, one containing the apex and the middle cochlear turn and the other the basal cochlear turn. The samples were placed into Nunclon Microwell plates (Sigma-Aldrich) filled with PBS and stored at 4°C. Later, the samples were permeabilized with 0.5% (v/v) Triton X-100 (Sigma-Aldrich) for 15 min at room temperature with shaking, and incubated for 1 h with a 1:1000 dilution of phalloidin (R Alexa Fluor 488, Invitrogen, Carlsbad, CA) in PBS. After three washes in PBS, the samples were transferred to slides, mounted with the aid of Vectashield mounting medium and stained with DAPI (Vector Laboratories, Burlingame, CA).[32]

*Image and statistical analyses*

Fluorescence intensities were analyzed in photographs obtained from three independent experiments using ZEN blue software (Zeiss, Jena, Germany). The data are presented as means ± standard errors of triplicate measurements. Statistical significance was identified by one-way analysis of variance (ANOVA) with Bonferroni *post hoc* test for ABR, and the *t*-test was used for all other results. A value of p of $< 0.05$ was taken to indicate statistical significance.

*Ethics approval*

All procedures were performed in accordance with national ethics guidelines. This study was approved by the Institutional Review Board of our hospital (approval no. CMCDJ-AP-2018-009).

## Results

*Preparation and characterization of ALA-NPs*

We fabricated ALA-NP by an oil-in-water emulsion method using Pluronic F-127 as a surfactant. Based on its hydrophobicity (log p = 2.18 calculated using ChemDraw), ALA was encapsulated into the soybean oil core of the NP and Pluronic F-127 formed the hydrophilic shell. The NPs were 187.3 ± 5.3 nm in diameter (Fig. 2a), and were spherical on TEM. The drug-encapsulation efficiency was ~ 86.54%, suggesting that loading was successful. The loading capacity of the NP was ~ 10.82%. We monitored the release of ALA from the NP for 64 h; < 20% of the ALA was released at 6 h and ALA release proceeded slowly thereafter, suggesting stable loading in the NP (Fig. 2b). The ALA-NP size and shape did not change significantly at 37° C, but ALA release was slightly higher than at room temperature (Fig. S1).

*In vitro antioxidant activity of ALA-NP in HEI-OC1 cells*

The cytotoxicity of ALA-NPs toward HEI-OC1 cells was evaluated by MTT assay. ALA-NPs at up to 5 mg/mL did not affect cell viability (Fig. 3a). Next, we evaluated whether pretreatment with ALA-NPs or free ALA protects against the effect of 5 mM kanamycin on HEI-OC1 cells; free ALA at 0.05–0.25 mg/mL and ALA-NPs at 0.25–5 mg/mL increased the viability of kanamycin-treated HEI-OC1 cells (Fig. 3b).

We analyzed the antioxidant effect of ALA-NPs and its dependence on the Nrf2/HO1 antioxidant pathway. First, as reported previously, we determined the effect of ALA and ALA-NPs on the nuclear translocation of NRF2 in HEI-OC1 cells.[33,34] Western blotting showed that the nuclear: cytoplasmic Nrf2 ratio was significantly increased by kanamycin treatment and was further increased by ALA or ALA-NPs (Fig. 4). There was no significant difference between the ALA and ALA-NP groups.

Next, we examined the effect of free ALA or ALA-NPs on the levels of antioxidant proteins in HEI-OC1 cells and the influence of Nrf2 inhibition on these effects. The addition of ALA-NPs prior

to kanamycin treatment significantly increased the NRF2, HO1, SOD1, and SOD2 levels compared to those of the kanamycin-alone and the ALA pretreated groups (Fig. 5a and 5b). The increases were significantly reduced by silencing of Nrf2 expression using siNrf2. Therefore, ALA-NP exerts an antioxidant effect via the Nrf2/HO1 antioxidant pathways.

When measuring ROS levels, DCF-DA served as a ROS sensor within HEI-OC1 cells. ROS production was increased by kanamycin treatment (Fig. 5c and 5d); this effect was reduced (to similar extents) by free ALA and ALA-NPs. Transfection of siNrf2 in the absence of ALA-NPs resulted in a similar increase in ROS production, suggesting that the antioxidant effect of ALA-NPs is mediated by Nrf2.

*RWM permeation efficiency of ALA-NPs*

To compare the mucosal permeation efficiencies of free ALA and ALA-NPs, an *in vitro* experiment was performed using an artificial mucosal membrane. ALA-NP membrane penetration was higher than that of free ALA (Fig. 6a). To explore the NP distribution *in vivo*, we injected NR-NP into the middle ear cavity and, 24 h later, examined the cochlea under a confocal microscope. Mice injected with NR-NP exhibited significantly more red fluorescence in the organ of Corti compared to controls; the dye was observed in both inner and outer hair cells (Fig. 6b and 6c).

*In vivo application*

Although 0.5–1 mg/mL ALA-NPs showed the greatest protective effect against ototoxicity *in vitro*, ALA-NPs at 5.0 or 2.5 mg/mL was injected into the middle ear because we considered loss of the drug during delivery from the middle ear to the cochlea, and these concentrations, showed no toxicity *in vitro*. The animals were divided into four groups and were administered saline, free ALA, 2.5 mg/mL ALA-NPs, or 5.0 mg/mL ALA-NPs at 4 h before the induction of toxicity. The free ALA

concentration was 0.5 mg/mL, which is equal to the concentration of ALA in 5.0 mg/mL ALA-NP.

To evaluate the auditory brainstem response (ABR), the hearing thresholds at various frequencies were measured in both groups. The lower the level of sound that can be heard, the better the hearing. At day 4 there were significant between-group differences in the ABR at 4 kHz ($p < 0.001$), 8 kHz ($p < 0.010$), 16 kHz ($p = 0.001$), 32 kHz ($p < 0.001$), and in the click test ($p < 0.001$) (Fig. 7a). The ALA-NP 5.0 mg/mL group exhibited significantly better hearing than the deaf-sham group at 4 kHz ($p < 0.001$), 8 kHz ($p = 0.012$), 16 kHz ($p = 0.001$), and 32 kHz ($p < 0.001$), and in the click test ($p < 0.001$) by Bonferroni *post hoc* test. However, the free ALA group exhibited better hearing than the deaf-sham group only in the click test ($p = 0.023$).

At day 7, there were significant between-group differences in hearing at 4 kHz ($p = 0.001$), 8 kHz ($p = 0.023$), 16 kHz ($p = 0.001$), and 32 kHz ($p = 0.001$) and in the click test ($p = 0.001$) (Fig. 7b). The ALA-NP 5.0 mg/mL group exhibited significantly better hearing than the deaf-sham group at 4 kHz ($p = 0.003$), 8 kHz ($p = 0.017$), 16 kHz ($p = 0.002$), 32 kHz ($p = 0.001$), and in the click test ($p = 0.002$) by Bonferroni *post hoc* test. The free-ALA group exhibited better hearing than the deaf-sham group at 16 kHz ($p = 0.009$) and in the click test ($p = 0.014$).

Western blotting showed that, in the ALA-NP group, the levels of NRF2, HO1, SOD1, and SOD2 were significantly increased compared to those of the deaf-sham group, and the levels of SOD1 and SOD2 were increased compared to those of the deaf-ALA group. Therefore, ALA-NPs activated the NRF2/HO1 antioxidant pathway *in vivo* (Fig. 8a and 8b). In addition, we observed the organ of Corti by confocal microscopy, focusing on four regions of the place-frequency map.[35] The stereocilia of the deaf-sham group were highly disrupted at 8, 16, 32, and 48 kHz, whereas those of the ALA-NP group were preserved at all four frequencies (Fig. 8c).

**Discussion**

The recovery rate from acute hearing loss is only about 60%, despite the use of high doses of systemic or intratympanic steroids.[36] Antioxidants may be an alternative therapeutic option. Here we delivered ALA in NPs to the cochlea.

*In vitro*, 0.25–5 mg/mL ALA-NPs inhibited the toxicity of kanamycin and increased cell viability. ALA-NPs contain 0.025–0.5 mg/mL of ALA; thus, the effective concentration range of ALA-NPs is somewhat wider than that of free ALA (0.05–0.25 mg/mL). This finding may have been caused by a toxic effect of the DMSO in the culture medium, or to more effective delivery of ALA by the ALA-NPs. Nonetheless, the data indicate that the ALA-NPs are safe and non-toxic.

We showed that NPs containing Nile red passed through the RWM and reached the organ of Corti (Fig. 6). In the mice with hearing loss, injection of 5 mg/mL ALA-NPs into the middle ear cavity yielded a significant therapeutic effect. Also, ALA-NPs were more effective than free ALA (Fig. 7). Unfortunately, we were unable to quantify the ALA concentration in the inner ear tissue. Generally, a large amount of drug is lost during transfer from the middle to the inner ear.[37] The concentration of ALA-NPs in the inner-ear tissue was likely to be < 1 mg/mL based on a prior report of drug concentration in the perilymph.[38] Use of a hydrogel to retain ALA-NPs in the middle ear and increase the duration of contact with the RWM, or use of a higher concentration of ALA-NPs would increase the concentration of drug in the inner ear, improving the outcomes of therapy.

In an inner ear cell line, ALA activated the Nrf2/HO1 antioxidant pathway; this effect was subsequently confirmed *in vivo*. This is the first report of the antioxidant activity of ALA in the inner ear of animals using NPs. The guidelines for the treatment of sudden hearing loss, a type of acute hearing loss, suggest that antioxidants do not benefit the patient.[14] However, in recent studies, antioxidant therapy for sudden hearing loss has shown positive results.[39-41] In most prior studies, antioxidants were administered systemically, probably resulting in low concentrations in the inner ear, with outcomes less successful than expected. Intratympanic injection overcomes this limitation, and a safe and effective inner ear drug-delivery system, such as ALA-NP, could yield better outcomes.

In summary, we encapsulated ALA (an antioxidant) within Pluronic F127-based NPs and delivered them to mice with hearing loss via intratympanic injection. The ALA-NPs were about 187.3 ± 5.3 nm in diameter, and ALA was stably loaded. Their safety and protective effects were evaluated in organ of Corti cells from HEI-OC1 mice. We observed nuclear translocation of NRF2 and increased levels of antioxidant proteins, including Nrf2, HO1, SOD1, and SOD2. When ALA-NPs were injected into the mouse middle ear cavity, hearing ability was significantly preserved after the induction of ototoxicity, compared to that of controls. Test cochleae exhibited increased levels of antioxidant proteins, as observed *in vitro*. Intratympanic injection of ALA-NPs effectively protected against acute hearing loss by activating the Nrf2/HO1 pathway.

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## Figures

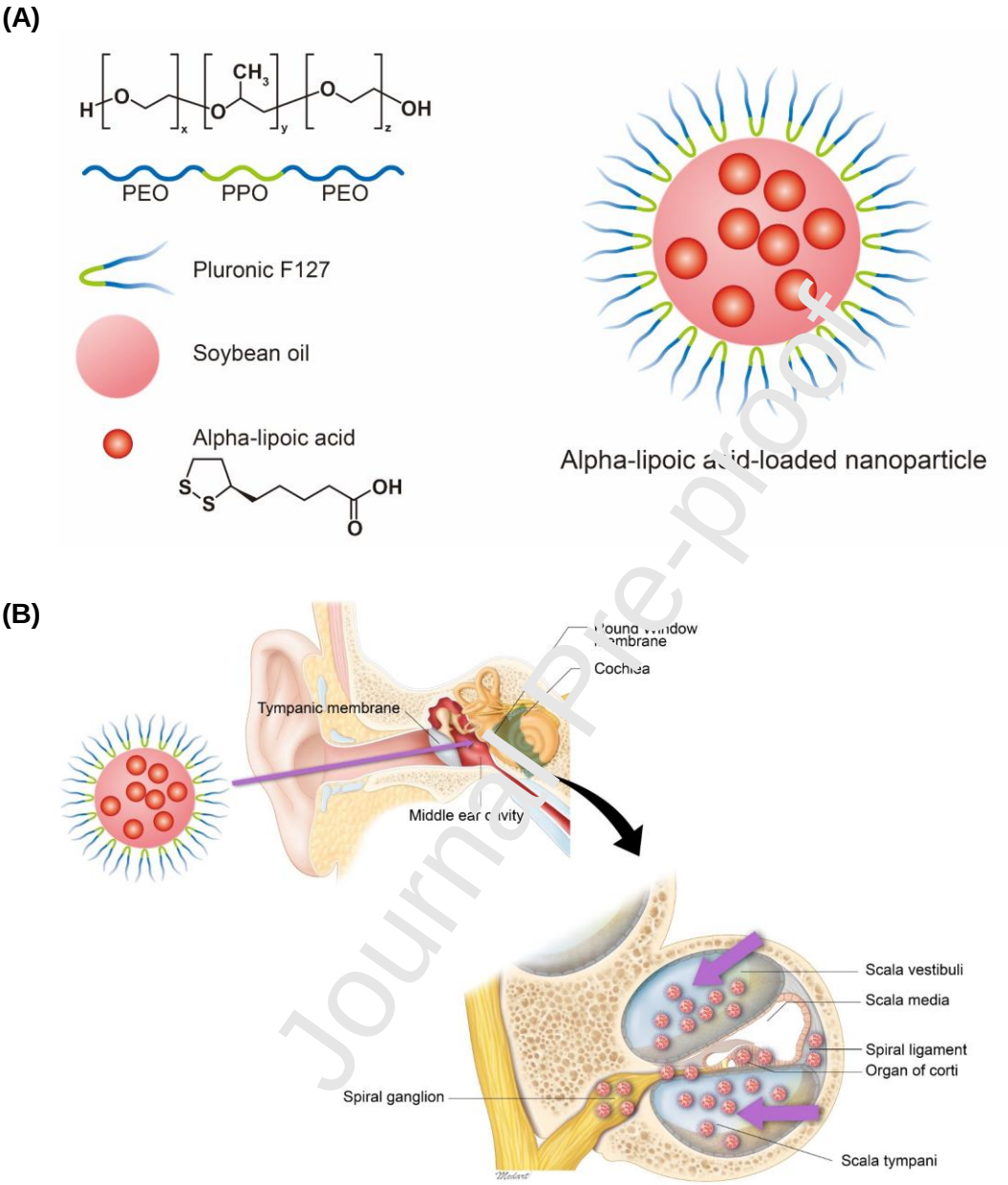


**Figure 1.** Schematic diagram of ALA-NPs produced using Pluronic F-127 (A) and their biodistribution after intratympanic administration (B).

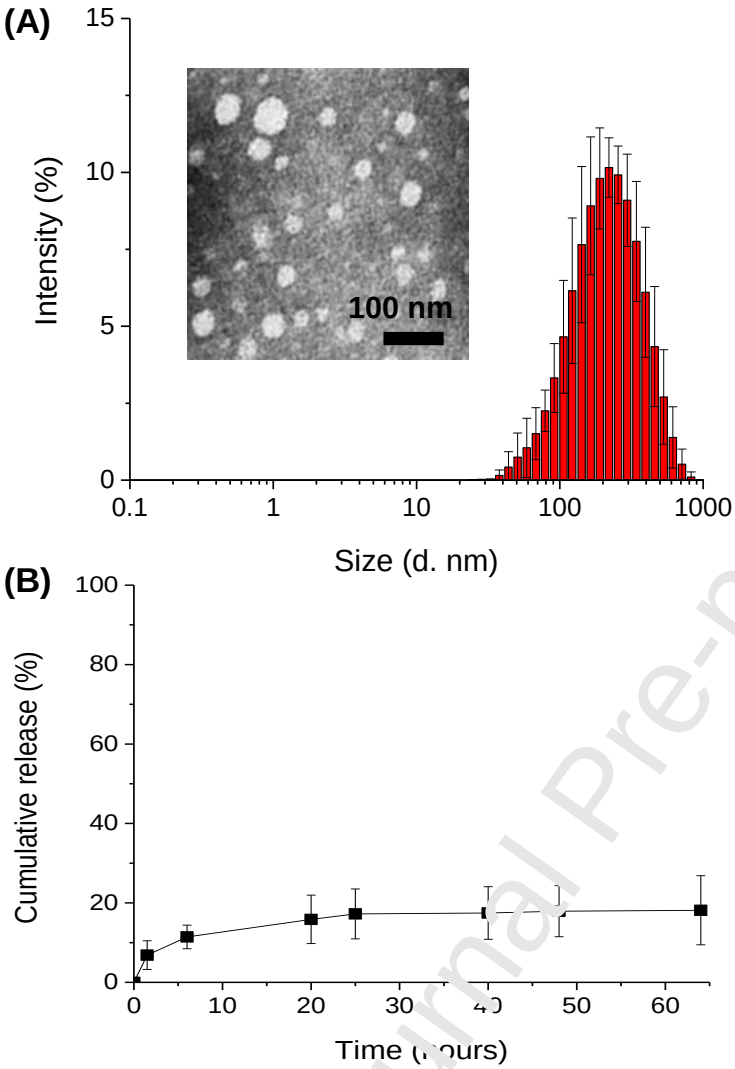


**Figure 2.** Characterization of ALA-NPs. (A) Size distribution of the ALA-NPs as determined using a Zetasizer (inset, TEM image of an ALA-NP). (B) Release of alpha-lipoic acid from the ALA-NPs over time.

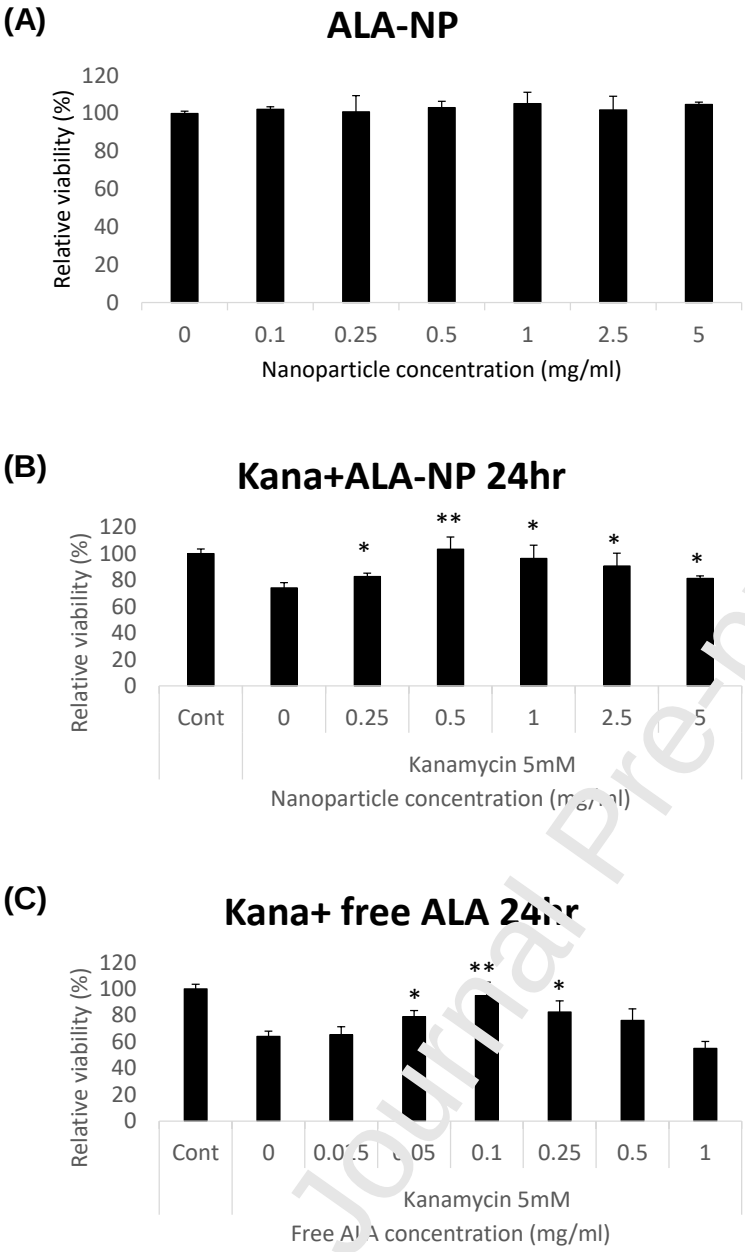


**Figure 3.** Cytotoxicity (A) and protective effects of ALA-NPs on kanamycin-induced ototoxicity in HEI-OC1 cells (B and C) by MTT assays. HEI-OC1 cells were exposed to 0.1–5 mg/mL ALA-NPs for 24 h, and no toxic effect was detected (A). HEI-OC1 cells were treated with 0.25–5 mg/mL ALA-NPs (B) or 0.025–1 mg/mL free ALA (C) for 1 h followed by 5 mM kanamycin for 24 h. ALA-NPs and free ALA protected against the cytotoxicity induced by 5 mM kanamycin (B).

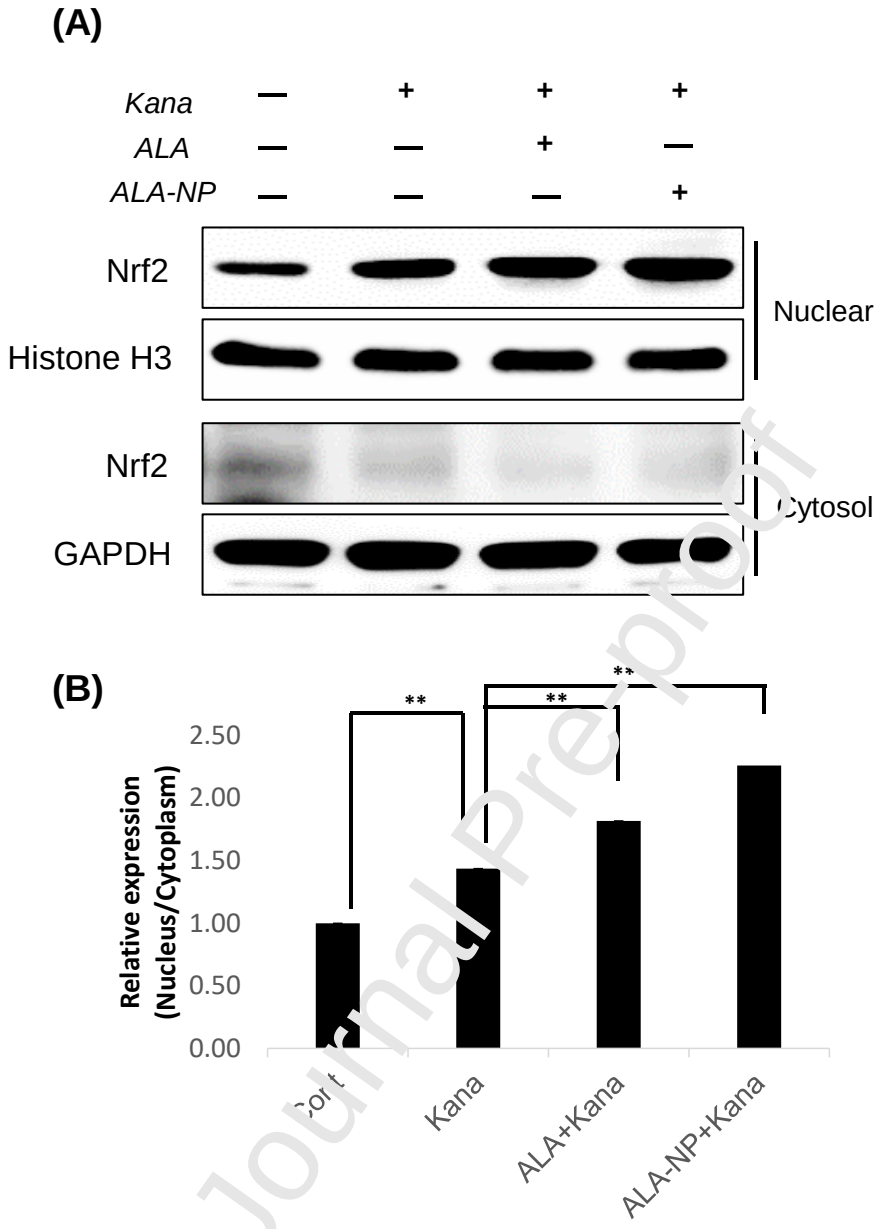


**Figure 4.** ALA and ALA-NPs promote the nuclear translocation of Nrf2. The relative nuclear to cytoplasmic NRF2 ratio was significantly increased by kanamycin and was further increased by treatment with ALA or ALA-NPs (A), (B). There was no significant difference between the ALA and ALA-NP groups. Data are means ± SD from three independent experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

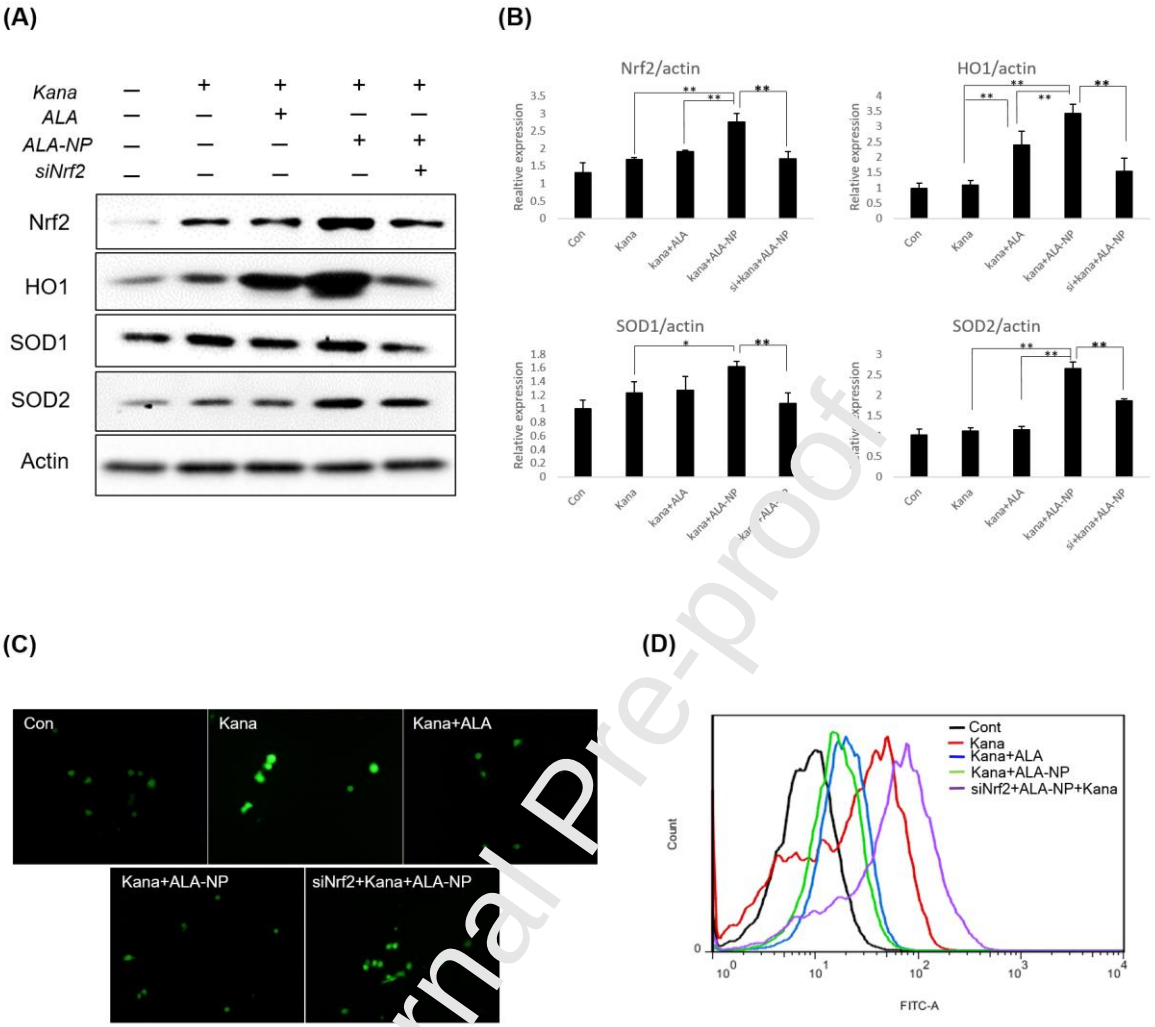


**Figure 5.** Effects of free ALA or ALA-NPs on the levels of intracellular antioxidant proteins (A), (B) ROS production (C), and (D) kanamycin-induced cytotoxicity in HEI-OC1 cells. HEI-OC1 cells were pretreated with 0.1 mg/mL free ALA or 1 mg/mL ALA-NPs for 24 h, followed by the application of 5 mM kanamycin. The addition of ALA-NPs prior to kanamycin treatment significantly increased the NRF2, HO1, SOD1, and SOD2 levels compared to the kanamycin-only and ALA-pretreated groups. This effect was reversed by inhibition of NRF2 (A), (B). Data are means ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ by independent *t*-test. si: siNRF2. Intracellular ROS level was measured by flow cytometry. The kanamycin-induced increased ROS level was reduced by free ALA and ALA-NPs (C), (D). Green fluorescence signals from cells were verified by fluorescence microscopy (C).

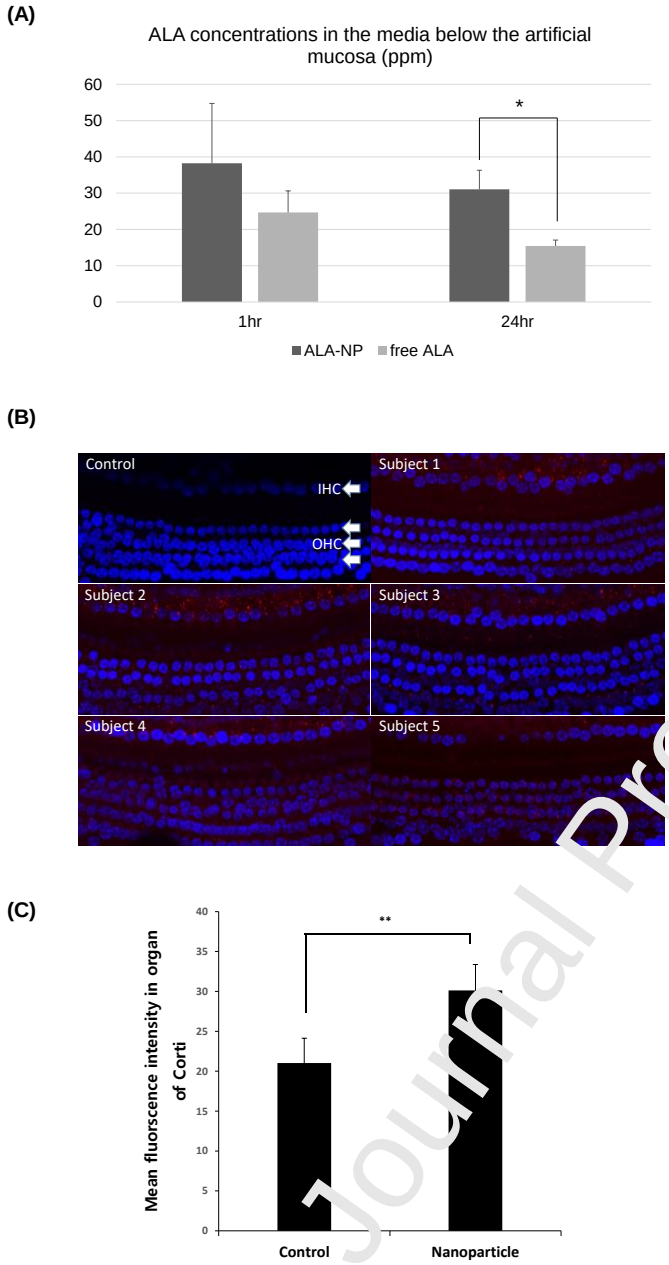




**Figure 6.** Permeation of the round window membrane by ALA-NPs. *In vitro* permeation experiments using an artificial mucosa. ALA-NPs penetrated the mucosa more efficiently than did free ALA at 1 and 24 h (A). Injection of Nile red-loaded NPs into the middle ear significantly increased the extent of red fluorescence in the organ of Corti compared to that of controls; dye was observed in both inner and outer hair cells 24 h after injection (B), (C).

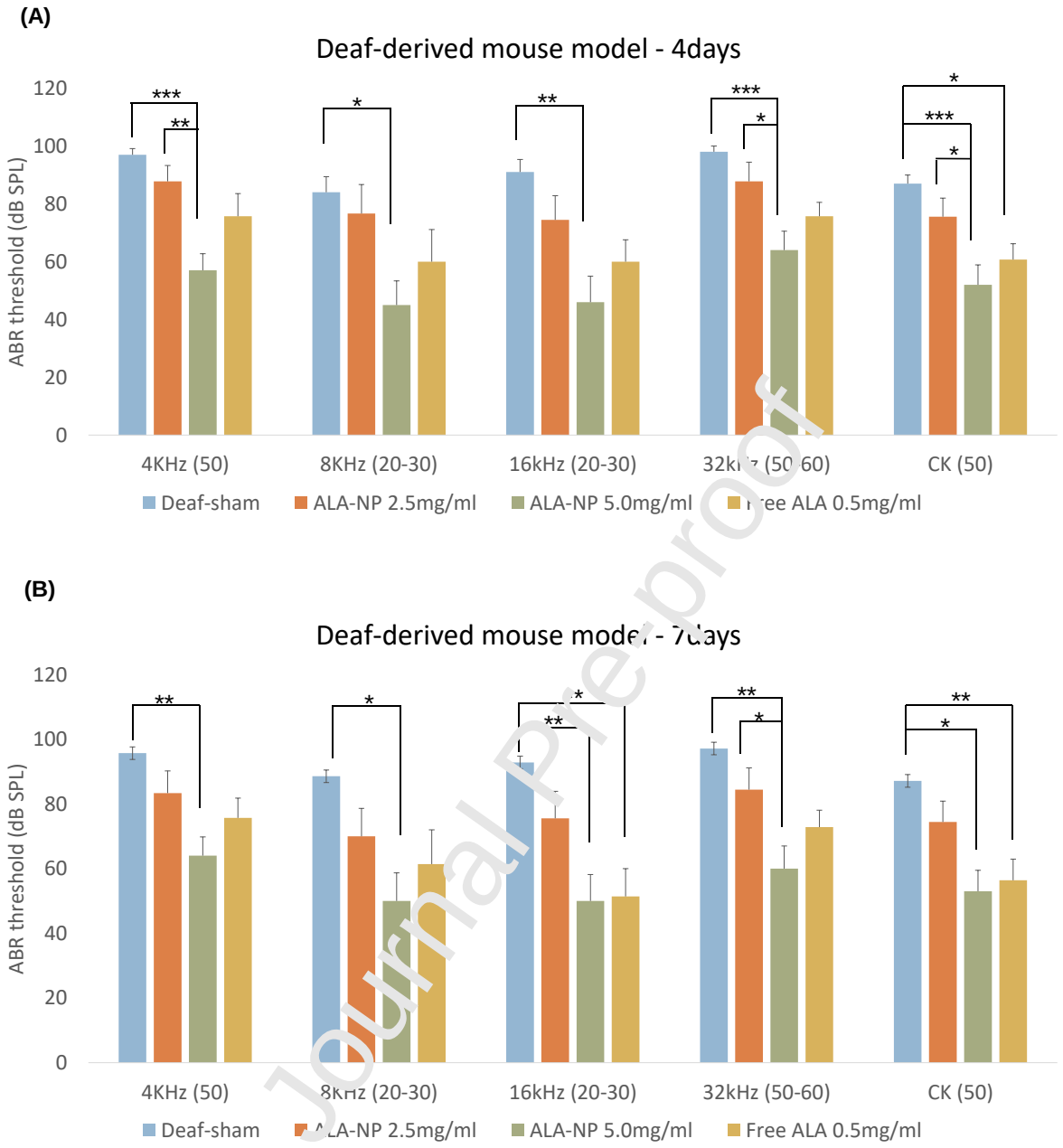


**Figure 7.** *In vivo* hearing preservation (A), (B). The ABR thresholds of the four groups were measured at days 4 (A) and 7 (B) after induction of deafness. Only the ALA-NP 5.0 mg/mL group exhibited significantly better hearing than the deaf-sham group at all frequencies tested. The statistical results are shown in the text. Data are means ± SD of three independent experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

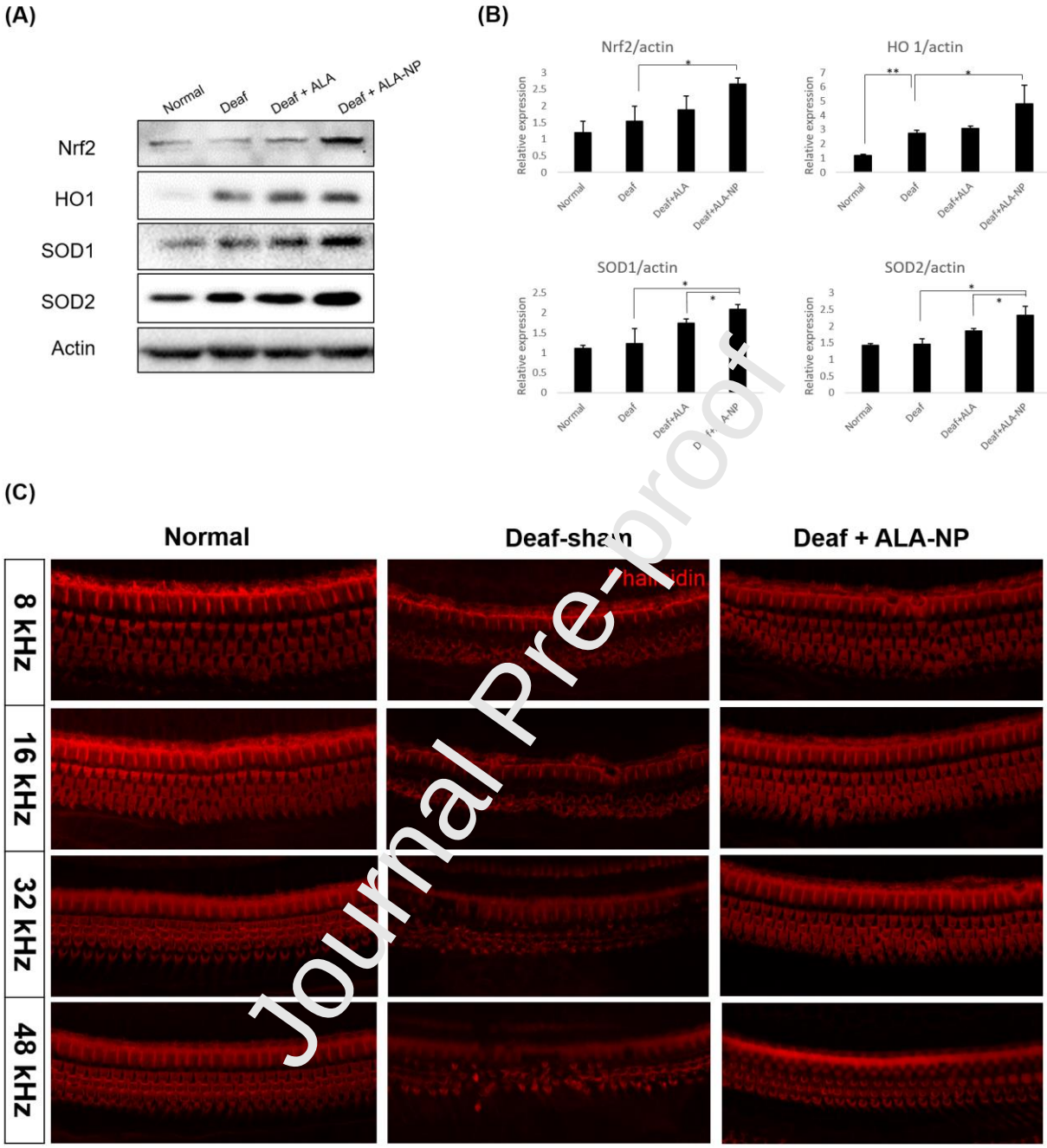




**Figure 8.** Therapeutic effect of ALA-NPs in the mouse model of ototoxicity. The levels of antioxidant proteins increased 72 h after induction of deafness in the ALA-NP group more so than in the control and ALA groups (A), (B). Data are means ± SD from three independent experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The organs of Corti of the deaf group exhibited severe loss of cilia and hair cells in all four frequency regions, although those in the deaf-ALA-NP group were preserved (C).

**Graphical Abstract (text)**

The purpose of this study was to develop a therapeutic method for acute hearing loss using an antioxidant-containing nanoparticle (NP). We selected alpha-lipoic acid (ALA) as an antioxidant and used pluronic F127 for fabrication of NPs. *In vitro* results showed the resulting ALA-loaded pluronic F127 nanoparticles (ALA-NPs) provided protective effects in HEI-OC1 mouse organ of Corti cells. ALA-NP also showed the nuclear translocation of NRF2 and an increase in antioxidant proteins. In animal study, the hearing of mice injected with ALA-NPs into the middle ear cavity was significantly preserved after ototoxicity induction compared to the control group. These results demonstrate that ALA-NPs showed effective hearing protection in acute hearing loss, which could be mediated through the Nrf2 / HO-1 pathway.

CRediT author statement

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