

Arginine–Dexamethasone Nanoplatfom Enables Biocatalytic Remodeling of the Cochlear Pathological Microenvironment

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Abstract

High-intensity noise exposure induces cochlear injury through mechanical trauma, disruption of ionic homeostasis, and secondary oxidative stress, ultimately leading to noise-induced hearing loss (NIHL). Increasing evidence suggests that oxidative stress not only causes direct cellular damage but also drives pathological remodeling of the cochlear microenvironment by triggering persistent inflammatory activation and impairing endogenous nitric oxide (NO) biocatalysis. However, current therapeutic strategies based solely on antioxidants or anti-inflammatory agents are insufficient to simultaneously restore inflammatory homeostasis and endogenous catalytic regulation within the injured cochlea. Here, we developed a carrier-free arginine–dexamethasone nanoplateform (AD NPs) through a one-step ultrasound-assisted self-assembly strategy to achieve biocatalytic remodeling of the pathological cochlear microenvironment. By integrating the anti-inflammatory activity of dexamethasone with the physiological substrate function of L-arginine for endothelial nitric oxide synthase (eNOS), AD was designed to coordinately suppress inflammatory signaling while restoring endogenous NO biocatalysis. The resulting nanoparticles exhibited an average diameter of approximately 169.9 nm, a positive surface charge (+ 17.18 mV), sustained ROS-responsive drug release, excellent biocompatibility, and prolonged cochlear retention following tympanic bulla administration. In H_2O_2 -induced cellular models, AD markedly reduced intracellular reactive oxygen species (ROS) accumulation, inhibited NF- κ B signaling activation, promoted macrophage polarization toward a pro-repair phenotype, restored eNOS/iNOS-associated NO biocatalytic homeostasis, improved endothelial functional phenotypes, and protected HEI-OC1 cells against oxidative injury. In a C57BL/6 mouse model of NIHL, AD significantly preserved high-frequency hearing, reduced cochlear hair cell loss, maintained ribbon synapse integrity, and corrected the pathological imbalance between eNOS and iNOS within the stria vascularis. Mechanistically, AD remodeled the pathological cochlear microenvironment through coordinated suppression of ROS/NF- κ B-mediated inflammatory activation and restoration of endogenous NO biocatalysis, thereby interrupting the reciprocal amplification between inflammatory dysregulation and vascular dysfunction following acoustic injury. Collectively, this work establishes a carrier-free biocatalytic nanoplateform that harnesses endogenous enzymatic regulation to remodel the pathological cochlear microenvironment. This strategy provides a mechanistic framework for microenvironment-oriented biocatalytic nanomedicine and offers a promising therapeutic approach for the treatment of noise-induced hearing loss.

1. Introduction

Noise-induced hearing loss (NIHL) is one of the most prevalent forms of acquired sensorineural hearing loss worldwide, resulting from either acute exposure to high-intensity noise or chronic occupational and environmental noise exposure.^[1, 2] As a major cause of permanent hearing impairment, NIHL severely compromises speech perception, communication ability, and quality of life, thereby imposing an increasing socioeconomic burden worldwide.^[3] Despite substantial advances in elucidating the pathological mechanisms underlying acoustic trauma, no clinically established therapy is currently

available to effectively prevent irreversible cochlear injury or restore auditory function after noise exposure.^[4] Because mammalian cochlear sensory hair cells and ribbon synapses possess extremely limited regenerative capacity, pathological alterations initiated during the early stages of acoustic injury frequently progress toward irreversible hearing impairment.^[5] Therefore, identifying therapeutic strategies capable of intervening in the early pathological events that drive cochlear degeneration has become a major objective in the development of disease-modifying therapies for NIHL.^[6, 7]

The pathogenesis of NIHL is a dynamic and multifactorial process involving mechanical trauma, disruption of ionic homeostasis, metabolic disturbance, and secondary oxidative stress. Among these pathological events, excessive accumulation of reactive oxygen species (ROS) is widely recognized as an early initiating factor of cochlear injury.^[8] However, increasing evidence indicates that ROS-mediated damage extends far beyond direct oxidative toxicity. Persistent oxidative stress activates NF- κ B-dependent inflammatory signaling, promotes macrophage polarization toward a pro-inflammatory phenotype, and disrupts endothelial nitric oxide synthase (eNOS)-dependent nitric oxide (NO) metabolism, thereby impairing vascular homeostasis within the cochlea.^[9] These interconnected pathological alterations cooperatively reshape the cochlear microenvironment into a self-perpetuating pathological state characterized by persistent inflammatory activation, endothelial dysfunction, and impaired endogenous NO biocatalysis. Consequently, the progression of NIHL is increasingly recognized as a process of pathological microenvironment remodeling rather than a consequence of oxidative injury alone. Therefore, therapeutic strategies capable of coordinately regulating inflammatory signaling and restoring endogenous catalytic homeostasis may provide greater therapeutic benefit than conventional interventions targeting a single pathological pathway.^[10]

Glucocorticoids, particularly dexamethasone (Dex), remain the first-line pharmacological intervention for acute inner-ear disorders owing to their potent anti-inflammatory properties.^[11] By suppressing the production of pro-inflammatory mediators, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and inducible nitric oxide synthase (iNOS), Dex effectively attenuates excessive inflammatory activation and provides partial protection against cochlear injury. Nevertheless, the therapeutic efficacy of Dex in NIHL remains limited because it primarily interrupts inflammatory signaling without correcting the accompanying disruption of vascular homeostasis and endogenous catalytic regulation within the pathological cochlear microenvironment. Consequently, although glucocorticoids alleviate inflammatory burden, they are insufficient to reverse the integrated pathological network established following acoustic trauma. These limitations highlight the need for therapeutic strategies that combine inflammatory regulation with restoration of endogenous microenvironmental homeostasis.^[11, 12]

Arginine (Arg), the physiological substrate of endothelial nitric oxide synthase (eNOS), plays a central role in endogenous nitric oxide biocatalysis and vascular homeostasis.^[13–19] Unlike exogenous NO donors that directly release nitric oxide, Arg supports physiological NO generation by fueling endogenous enzymatic catalysis, thereby preserving vascular function while minimizing uncontrolled nitrosative stress.^[20] Increasing evidence has demonstrated that supplementation with Arg restores

endothelial responsiveness, improves NO bioavailability, and alleviates vascular dysfunction in multiple oxidative stress-associated diseases.^[21–26] These characteristics make Arg an attractive biocatalytic component for regulating pathological microenvironments through endogenous enzymatic mechanisms rather than direct pharmacological intervention. Therefore, incorporating Arg into a locally delivered nanoplatform provides the opportunity to restore impaired NO biocatalysis while complementing conventional anti-inflammatory therapy.

Based on the complementary biological functions of Dex and Arg, we engineered a carrier-free arginine–dexamethasone self-assembled nanoplatform through a simple ultrasound-assisted assembly strategy. Rather than functioning solely as a drug delivery system, AD was designed as a biocatalytic nanoplatform capable of coordinately suppressing inflammatory activation and restoring endogenous NO biocatalysis within the pathological cochlear microenvironment. We hypothesized that simultaneous regulation of these two interconnected pathological axes would interrupt the reciprocal amplification between inflammatory dysregulation and vascular dysfunction, thereby remodeling the pathological cochlear microenvironment and providing superior protection against noise-induced cochlear injury.

To validate this hypothesis, we systematically investigated the physicochemical properties, cochlear delivery behavior, biological activities, and therapeutic efficacy of AD in both cellular and murine models of NIHL. Furthermore, we explored whether endogenous biocatalytic regulation mediated by the AD nanoplatform contributes to pathological microenvironment remodeling, thereby providing mechanistic insight into microenvironment-oriented biocatalytic nanomedicine for the treatment of noise-induced hearing loss.

2. Materials and Methods

2.1 Materials and Reagents

Arg, Dex, hydrogen peroxide (H_2O_2), and other routine reagents were purchased from Aladdin (Shanghai, China). Methanol, absolute ethanol, and other hazardous chemicals were procured through the Translational Medicine Center of Ruian People's Hospital, which managed their centralized purchasing and distribution. Cell Counting Kit-8 (CCK-8), the Calcein-AM/PI Cell Viability and Cytotoxicity Assay Kit, ROS Assay Kit, Hoechst 33342, Bicinchoninic Acid (BCA) Protein Assay Kit, bovine serum albumin (BSA), and DAPI were obtained from Beyotime Biotechnology (Shanghai, China). EDTA decalcification solution and the NO fluorescent probe DAF-FM DA were purchased from Biosharp (Anhui, China). Donkey serum was obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) and cell culture-related reagents were purchased from Gibco (California, USA) and Servicebio (Wuhan, China), respectively.

Primary antibodies used for Western blot analysis included rabbit anti-Ptch1, Gli1, HO-1, I κ B α , phospho-I κ B α (p-I κ B α), p65, p50, eNOS, iNOS, and VEGFA. Primary antibodies employed for immunofluorescence staining included Arg-1, iNOS, HO-1, VEGFA, Ptch1, Gli1, Myosin VIIa, CtBP2, and GluR2. The

corresponding fluorescently labeled secondary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Thermo Fisher Scientific, Affinity Biosciences, or other commercial suppliers. Alexa Fluor 488-conjugated phalloidin used for cytoskeletal staining was obtained from Thermo Fisher Scientific, and antifade mounting medium was purchased from DAKO. Unless otherwise specified, all reagents were used as received without further purification, and all experimental solutions were freshly prepared immediately before use.

2.2 Preparation of AD Self-Assembled Nanoparticles

AD nanoparticles were prepared using a one-step ultrasound-assisted self-assembly method. Briefly, 50 mg of Dex was dissolved in 3 mL of methanol and sonicated at room temperature for 30 min to obtain a homogeneous solution. The resulting Dex solution was then added dropwise to 30 mL of ultrapure water containing 50 mg of Arg under continuous magnetic stirring at 500 rpm. The mixture was subsequently sonicated in an ice bath for 4 h to facilitate the formation of stable self-assembled nanoparticles through electrostatic interactions, hydrogen bonding, and hydrophobic interactions between Arg and Dex. Following sonication, the suspension was centrifuged at 10,000 rpm for 30 min, after which the supernatant was discarded and the precipitate was collected. The precipitate was redispersed in ultrapure water, pre-frozen at -80°C overnight, and lyophilized for 48 h to obtain dry AD nanoparticle powder. Residual methanol was effectively removed during the centrifugation, redispersion, and subsequent freeze-drying processes.

To optimize the physicochemical properties of the nanoparticles, three formulations with Arg:Dex mass ratios of 1:2, 1:1, and 2:1 were prepared. The optimal formulation was selected for subsequent experiments based on its particle size, polydispersity index (PDI), and zeta potential.

2.3 Physicochemical Characterization

The morphology of the AD nanoparticles was characterized using transmission electron microscopy (TEM; Tecnai G2 F20, FEI, USA). Briefly, the nanoparticle suspension was diluted with ultrapure water, deposited onto carbon-coated copper grids, allowed to air-dry, and subsequently imaged. The hydrodynamic particle size, PDI, and zeta potential were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Panalytical, UK) at 25°C . Each sample was analyzed in triplicate, and the mean values were calculated.

Ultraviolet–visible (UV–Vis) absorption spectra of Arg, Dex, and AD were acquired using a UV–Vis spectrophotometer (UV-2600, Shimadzu, Japan) over a wavelength range of 190–400 nm. The surface elemental composition and chemical states of AD were further characterized by X-ray photoelectron spectroscopy (XPS; Nexsa X, Thermo Fisher Scientific, USA). All binding energies were calibrated against the C1s reference peak at 284.8 eV.

2.4 In Vitro Drug Release

The in vitro release profile of AD was evaluated using a dialysis-based method. Briefly, 5 mg of AD was dispersed in 1 mL of PBS, (pH 7.4), loaded into dialysis bags with a molecular weight cutoff (MWCO) of

3500 Da, and immersed in 20 mL of either PBS or PBS supplemented with 400 μM H_2O_2 to simulate an oxidative microenvironment. The dialysis bags were incubated at 37°C with continuous shaking at 100 rpm. At predetermined time points (0.5, 1, 2, 4, 8, 12, 24, 48, and 72 h), 1 mL of the release medium was withdrawn and immediately replaced with an equal volume of fresh medium to maintain sink conditions.

The concentrations of the released drug were quantified by UV–visible spectrophotometry. The cumulative release percentage was subsequently calculated using a standard calibration curve to characterize the time-dependent release kinetics and ROS-responsive release behavior of AD.

2.5 Hemolysis Assay

The hemocompatibility of AD was evaluated using a red blood cell (RBC) hemolysis assay. Fresh mouse whole blood was collected in EDTA-containing anticoagulant tubes and centrifuged to isolate the RBCs. The collected RBCs were washed three times with PBS and subsequently diluted with PBS to prepare a 2% (v/v) RBC suspension. Equal volumes of the RBC suspension and AD solutions at final concentrations of 0.1, 0.25, 0.5, 1, and 2 mg mL^{-1} were mixed and incubated at 37°C for 2 h. PBS and ultrapure water served as the negative and positive controls, respectively.

Following incubation, the samples were centrifuged to pellet the intact RBCs, and the supernatants were collected for analysis. The absorbance of each supernatant was measured at 540 nm using a microplate reader, and the hemolysis rate was calculated relative to the negative and positive control groups. A hemolysis rate of less than 5% was considered indicative of acceptable hemocompatibility.

2.6 Cell Culture and Establishment of the Oxidative Stress Model

RAW264.7 mouse macrophages, HEI-OC1 mouse cochlear hair cell-like cells, and human umbilical vein endothelial cells (HUVECs) were obtained from Shanghai Fuheng Biotechnology (Shanghai, China). RAW264.7 cells and HUVECs were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO_2 . HEI-OC1 cells were maintained in the same culture medium at 33°C under an atmosphere of 10% CO_2 . The culture medium was refreshed every 1–2 days, and cells were passaged upon reaching approximately 70%–80% confluence.

An in vitro oxidative stress model was established by exposing cells to 400 μM H_2O_2 . The experimental groups consisted of the Control, H_2O_2 , H_2O_2 + Arg, H_2O_2 + Dex, and H_2O_2 + AD groups. Based on the results of the CCK-8 assay, the working concentration of AD was set at 100 $\mu\text{g mL}^{-1}$. Free Arg and Dex were administered at doses equivalent to the amounts contained in the AD formulation. Specifically, the concentrations of free Arg and Dex were calculated according to their respective mass fractions within AD to ensure dose equivalence across all treatment groups.

2.7 In Vitro Cytotoxicity of AD

RAW264.7 cells, HEI-OC1 cells, and HUVECs were seeded into 96-well plates at a density of 5×10^3 cells per well and allowed to adhere overnight. The cells were subsequently treated with AD at final concentrations of 1, 3, 5, 10, 20, 30, 40, 50, 75, and 100 $\mu\text{g mL}^{-1}$ for 24 h. Following treatment, 10 μL of CCK-8 working solution was added to each well, and the plates were incubated at 37°C for an additional 30 min. The absorbance at 450 nm was measured using a BioTek Synergy H1 microplate reader (BioTek, USA). Cell viability was expressed as the percentage of the absorbance value of the treated group relative to that of the untreated control group.

2.8 Intracellular ROS Detection

Based on the results of the in vitro cytotoxicity assay, 100 $\mu\text{g mL}^{-1}$ was selected as the working concentration of AD for all subsequent cell experiments. RAW264.7 cells were seeded into 24-well plates at a density of 2×10^4 cells per well and allowed to adhere for 12 h. The cells were then assigned to the following experimental groups: Control, H_2O_2 , H_2O_2 + Arg, H_2O_2 + Dex, and H_2O_2 + AD. Oxidative stress was induced by treatment with H_2O_2 at a final concentration of 400 μM . After 4 h of treatment, the cells were washed three times with PBS and incubated with 10 μM DCFH-DA working solution at 37°C in the dark for 30 min. Following incubation, the cells were washed three additional times with PBS to remove excess probe. Intracellular ROS fluorescence was then visualized using a laser scanning confocal microscope (Leica Stellaris 5 SR, Germany). Five randomly selected fields of view were analyzed for each group, and the mean fluorescence intensity (MFI) was quantified using ImageJ software.

2.9 Detection of Macrophage Inflammatory Response and Polarization

RAW264.7 cells were seeded into 6-well plates at a density of 5×10^5 cells per well and cultured overnight at 37°C . The cells were subsequently exposed to H_2O_2 and treated with the indicated formulations according to the experimental grouping described above. Following treatment, cells were harvested for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analyses. RT-qPCR was performed to quantify the expression of inflammation- and macrophage polarization-related genes, including TNF- α , IL-1 β , IL-6, iNOS, Arg-1, IL-10, and CD206.

For immunofluorescence analysis, cells were fixed, permeabilized, and blocked before incubation with primary antibodies against iNOS and Arg-1, followed by the appropriate fluorescent secondary antibodies. Macrophage polarization was subsequently assessed using laser scanning confocal microscopy. Western blot analysis was performed to evaluate the expression of key proteins involved in the NF- κB signaling pathway, including I $\kappa\text{B}\alpha$, phosphorylated I $\kappa\text{B}\alpha$ (p-I $\kappa\text{B}\alpha$), p65, and p50.

2.10 Transcriptome Sequencing and Bioinformatics Analysis

Total RNA was extracted from RAW264.7 and HEI-OC1 cells following the indicated treatments. RNA quality and integrity were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA), and

only samples with an RNA integrity number (RIN) ≥ 7.0 were used for subsequent library preparation. RNA library construction and high-throughput sequencing were performed by Paysono Biotechnology Ltd. (Shanghai, China).

Differentially expressed genes (DEGs) were identified using the thresholds of $|\log_2 \text{fold change}| \geq 1$ and an adjusted p value < 0.05 . Global transcriptomic alterations were visualized by principal component analysis (PCA), volcano plots, and heatmaps. To further elucidate the biological functions and molecular mechanisms regulated by AD, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) were performed to identify significantly enriched biological processes and signaling pathways.

2.11 Western Blot Analysis

Following the indicated treatments, total cellular proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentrations were quantified using a BCA Protein Assay Kit. Equal amounts of protein (30 μg per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated overnight at 4°C with the appropriate primary antibodies. After thorough washing, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were detected using an enhanced chemiluminescence (ECL) imaging system, and band intensities were quantified with ImageJ software. β -Actin was used as the internal loading control for normalization.

2.12 Endothelial Function and NO Detection

Following oxidative stress induction, intracellular NO levels in HUVECs were measured using the fluorescent probe DAF-FM DA at a final concentration of 5 μM . Endothelial angiogenic capacity was evaluated using a Matrigel tube formation assay. Briefly, HUVECs (1×10^4 cells per well) were seeded onto Matrigel-coated plates, incubated at 37°C for 6 h, and subsequently imaged. Angiogenic parameters, including total tube length, branch number, and node number, were quantified using the Angiogenesis Analyzer plugin in ImageJ.

A scratch wound healing assay was performed to assess endothelial cell migratory capacity. Briefly, a linear scratch was created in the cell monolayer using a sterile 200 μL pipette tip, and images were captured immediately after scratching (0 h) and after 24 h of incubation. The wound closure rate was subsequently calculated to evaluate cell migration. In addition, vascular endothelial growth factor A (VEGFA) expression was analyzed by immunofluorescence staining, RT-qPCR, and Western blot analysis.

2.13 HEI-OC1 Cell Protection Assay

HEI-OC1 cells were exposed to H_2O_2 -induced oxidative stress and subsequently treated with Arg, Dex, or AD. Cell viability was first evaluated using the CCK-8 assay. Cellular survival was further assessed using a Calcein-AM/PI Live/Dead Cell Viability Assay, in which Calcein-AM-positive (green) fluorescence

represented viable cells, whereas propidium iodide (PI)-positive (red) fluorescence indicated dead cells. Cytoskeletal organization and cellular morphology were examined by staining with Alexa Fluor 488-conjugated phalloidin. To evaluate the cellular oxidative stress response, heme oxygenase-1 (HO-1) expression was analyzed by immunofluorescence staining. In addition, the expression of Patched 1 (Ptch1) and glioma-associated oncogene homolog 1 (Gli1) was assessed by both immunofluorescence staining and Western blot analysis to investigate the modulation of Hedgehog signaling-associated cytoprotective pathways.

2.14 Immunofluorescence Staining

Cells or cochlear tissue samples were fixed in 4% paraformaldehyde and washed three times with PBS. The samples were then permeabilized with 0.3% Triton X-100 and blocked with 5% BSA for 1 h at room temperature to minimize nonspecific binding. Subsequently, the samples were incubated overnight at 4°C with the appropriate primary antibodies. After thorough washing with PBS, the samples were incubated with the corresponding Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature in the dark. Cell nuclei were counterstained with DAPI before mounting. Fluorescence images were captured using a Leica Stellaris 5 laser scanning confocal microscope, and fluorescence intensity was quantified using ImageJ software.

2.15 Establishment of the Mouse NIHL Model and Drug Administration

Eight-week-old male C57BL/6J mice (20–25 g) were purchased from SPF Biotechnology Co., Ltd. (Beijing, China). All animal experiments were approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (Approval No. ZN2025294) and conducted in accordance with the national guidelines for the care and use of laboratory animals. Prior to the experiments, all mice underwent auditory brainstem response (ABR) testing to confirm normal baseline hearing. Eligible animals were randomly allocated into five experimental groups (n = 5 per group): Control, Noise, Noise + Arg, Noise + Dex, and Noise + AD.

Drug administration was performed one day before noise exposure via tympanic bulla injection. Briefly, mice were anesthetized with 2% isoflurane delivered by inhalation and positioned on a thermostatically controlled surgical platform. Following routine aseptic disinfection, a postauricular incision was made to expose the tympanic bulla under an operating microscope. AD was freshly prepared in sterile saline at a concentration of 5 mg mL⁻¹. Free Arg and Dex solutions were prepared according to their respective proportions in the AD formulation to ensure dose equivalence among the treatment groups. A total volume of 10 µL of AD, Arg, or Dex solution was slowly injected into the tympanic bulla using a microsyringe. In the AD-treated group, this corresponded to a dose of 50 µg per ear. After injection, the incision was carefully closed, and the treated ear was maintained in an upward-facing position for approximately 30 min to facilitate drug retention within the middle ear cavity and enhance diffusion across the round window membrane (RWM). Mice in the Control and Noise groups underwent the identical surgical procedure but received an equivalent volume of sterile saline.

Noise exposure was induced using broadband noise (2–20 kHz) generated by a TDT RZ6 Multi-I/O Processor (Tucker-Davis Technologies, USA) and delivered through a loudspeaker connected to a power amplifier. Mice were placed in a custom-built sound-attenuating chamber and exposed to broadband noise at 110 dB SPL for 2 h, whereas animals in the Control group were not subjected to acoustic stimulation. The sound pressure level was continuously monitored and calibrated using a sound level meter to ensure stable exposure conditions throughout the experiment. ABR thresholds were measured at 3, 7, and 14 days after noise exposure. Following the final auditory assessment, the mice were euthanized, and cochlear tissues were harvested for subsequent histological and molecular analyses.

2.16 ABR Measurement

ABR thresholds were measured using a TDT RZ6 auditory electrophysiology workstation (Tucker-Davis Technologies, USA). Baseline ABR recordings were obtained before noise exposure to confirm normal hearing in all animals, and follow-up measurements were performed on days 3, 7, and 14 after noise exposure to assess temporal changes in auditory function. Prior to each recording session, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg⁻¹) and placed on a thermostatically controlled heating pad maintained at 37°C to preserve body temperature throughout the procedure.

Subdermal needle electrodes were inserted at the vertex (recording electrode), the postauricular mastoid region of the tested ear (reference electrode), and the contralateral hind limb (ground electrode). Tone-burst stimuli at frequencies of 8, 16, and 32 kHz were presented to the tested ear. Stimulus intensity was initially set at 90 dB SPL and then decreased in 10 dB increments until reproducible ABR waveforms were no longer detectable. At each stimulus intensity, responses were averaged over 512 sweeps to improve the signal-to-noise ratio.

The ABR threshold was defined as the lowest sound pressure level at which a characteristic and reproducible waveform could be visually identified. Thresholds were independently determined by two investigators blinded to the experimental groups. In cases of disagreement, the recordings were jointly re-examined until a consensus threshold was reached. Identical recording parameters, electrode placement, and testing procedures were maintained across all experimental groups and time points to ensure the consistency and reliability of the measurements.

2.17 Cochlear Whole-Mount Preparation and Immunofluorescence Staining of Hair Cells and Ribbon Synapses

Following the final ABR assessment, mice were deeply anesthetized and transcardially perfused with PBS, followed by 4% paraformaldehyde. The cochleae were carefully dissected and post-fixed in 4% paraformaldehyde at 4°C for 12 h. After fixation, the specimens were decalcified in 10% EDTA for 72 h, with the decalcification solution replaced daily. The basilar membrane was subsequently microdissected

under a stereomicroscope to prepare cochlear whole-mount specimens. The tissues were permeabilized with 0.5% Triton X-100 for 30 min and blocked with 10% donkey serum for 1 h at room temperature.

For hair cell analysis, cochlear whole mounts were incubated overnight at 4°C with rabbit anti-Myosin VIIa primary antibody, followed by incubation with the corresponding fluorescent secondary antibody. Alexa Fluor 488-conjugated phalloidin was used to label filamentous actin (F-actin) and visualize hair cell morphology. For ribbon synapse analysis, the tissues were incubated with mouse anti-CtBP2 (IgG1) and mouse anti-GluR2 (IgG2a) primary antibodies, followed by subclass-specific fluorescent secondary antibodies. Triple immunofluorescence staining was performed by sequential incubation with antibodies against CtBP2, GluR2, and Myosin VIIa, followed by the corresponding species- or subclass-specific fluorescent secondary antibodies. All primary antibody incubations were carried out overnight at 4°C, whereas secondary antibody incubations were performed in the dark according to the manufacturers' protocols.

Following mounting with antifade mounting medium, fluorescence images were acquired using a Leica Stellaris 5 laser scanning confocal microscope (Leica Microsystems, Germany). Hair cell survival was evaluated in the apical, middle, and basal turns of the cochlea, representing the low-, middle-, and high-frequency regions, respectively. Outer hair cells (OHCs) were counted within a standardized 200-μm segment from each cochlear region for every animal, and OHC survival was expressed as the percentage of surviving cells relative to the expected number of OHCs within the corresponding segment. Ribbon synapses were quantified in inner hair cells (IHCs) from the same cochlear regions. For each region, at least six consecutive IHCs per animal were analyzed. Co-localized CtBP2- and GluR2-positive puncta were defined as functional ribbon synapses, and the number of paired puncta per IHC was quantified using ImageJ software. All image acquisition and quantitative analyses were conducted by investigators blinded to the experimental group assignments to minimize observational bias.

2.18 In Vivo Distribution and Cochlear Retention of AD

To evaluate the cochlear delivery efficiency and local retention of AD, the near-infrared fluorescent dye Cy7 was physically adsorbed onto the surface of AD nanoparticles to generate Cy7-labeled AD (Cy7-AD). Briefly, AD was dispersed in sterile saline and incubated with a Cy7 solution under gentle stirring in the dark to facilitate the physical adsorption of Cy7 onto the nanoparticle surface. The resulting suspension was centrifuged to remove unbound Cy7, after which the nanoparticle pellet was washed, redispersed in sterile saline, and either used immediately or stored protected from light until administration.

Cy7-AD was administered by tympanic bulla injection one day before imaging, following the procedure described above. In vivo fluorescence imaging was performed on days 1, 3, 7, and 14 after administration using an IVIS Spectrum imaging system (PerkinElmer, USA). Fluorescence signals within the middle ear and cochlear regions were recorded at each time point to monitor the distribution and retention of the nanoparticles. At the designated time points, mice were euthanized, and the cochleae, heart, liver, spleen, lungs, and kidneys were harvested for ex vivo fluorescence imaging. Fluorescence intensity was quantified using Living Image software (PerkinElmer, USA), and the relative fluorescence

intensity within the cochlear region was calculated to evaluate the cochlear delivery efficiency and local retention profile of AD following tympanic bulla injection.

2.19 RT-qPCR Analysis of Cochlear Tissues

Following the completion of all endpoint assessments, mice were euthanized, and cochlear tissues were rapidly dissected, snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using HiScript III RT SuperMix (Vazyme, China), and quantitative real-time PCR (RT-qPCR) was subsequently performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China).

The expression levels of TNF- α , IL-1 β , IL-6, iNOS, Arg-1, IL-10, and CD206 were quantified, with GAPDH serving as the internal reference gene. The PCR amplification protocol consisted of an initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

2.20 Immunohistochemical Staining of Cochlear Tissues

Fixed and decalcified cochlear tissues were embedded in paraffin and sectioned into 4- μm -thick serial sections. The sections were deparaffinized, rehydrated through a graded ethanol series, and subjected to heat-induced antigen retrieval in sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide, after which the sections were blocked with 5% BSA for 1 h at room temperature to minimize nonspecific binding.

The sections were incubated overnight at 4°C with primary antibodies against eNOS or iNOS. After washing with PBS, the sections were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive signals were visualized using a 3,3'-diaminobenzidine (DAB) chromogenic detection system. The sections were subsequently counterstained with hematoxylin, dehydrated, and coverslipped. Bright-field images were acquired using an Olympus BX53 microscope (Olympus, Japan).

The stria vascularis was designated as the region of interest (ROI) for quantitative analysis. For each animal, three representative cochlear sections were selected, and three randomly chosen fields within the stria vascularis were analyzed from each section. Positive immunohistochemical staining was quantified using ImageJ software. Identical threshold parameters were applied to all images to ensure consistent analysis across groups. The integrated optical density (IOD), calculated as the product of the positively stained area and the mean optical density within the ROI, was used to quantify protein expression. All quantitative analyses were performed by investigators blinded to the experimental group assignments.

2.21 Histological and Biosafety Evaluation

Major organs, including the heart, liver, spleen, lungs, and kidneys, were harvested from mice in each experimental group for histopathological evaluation. The tissues were fixed in 4% paraformaldehyde, dehydrated through a graded ethanol series, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) according to standard histological procedures. Tissue architecture, inflammatory cell infiltration, necrosis, and other pathological abnormalities were examined under a light microscope to assess the in vivo biosafety and systemic biocompatibility of AD.

2.22 Statistical Analysis

All statistical analyses were performed using GraphPad Prism and R software. Data are presented as the mean $\pm$ standard deviation (SD). The sample size (n) and the number of biological replicates for each experiment are specified in the corresponding figure legends. Unless otherwise stated, n represents the number of independent biological replicates in the in vitro experiments. For animal studies, n denotes the number of animals, cochleae, or biological specimens analyzed, as indicated in the respective figure legends. Data normality was assessed before conducting parametric statistical analyses when appropriate.

Comparisons between two groups were performed using an unpaired two-tailed Student's t -test. Comparisons involving three or more groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple-comparison test. For ABR threshold data obtained across multiple frequencies and/or time points, two-way ANOVA or repeated-measures two-way ANOVA was performed as appropriate, followed by the corresponding post hoc multiple-comparison tests. For RNA sequencing (RNA-seq) analysis, P values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate correction. Genes with an absolute $\log_2$ fold change ($|\log_2\text{FC}| \geq 1$) and an adjusted P value < 0.05 were considered differentially expressed. A two-sided P value < 0.05 was considered statistically significant throughout the study.

3. Results

3.1 Design Rationale and Construction of the AD Biocatalytic Nanoplatfom

The design rationale, fabrication strategy, local delivery pathway, and proposed therapeutic mechanism of the AD nanoplatfom for the treatment of NIHL are summarized in Fig. 1. Acoustic trauma initiates a cascade of pathological events, including mechanical injury, disruption of ionic homeostasis, excessive ROS accumulation, inflammatory activation, and impaired endogenous NO regulation, ultimately contributing to the establishment of a pathological cochlear microenvironment. Therefore, a therapeutic strategy capable of simultaneously regulating inflammatory imbalance and restoring endogenous catalytic homeostasis may provide an effective approach for microenvironment remodeling after noise exposure.

Based on this concept, Dex and Arg were rationally integrated into a carrier-free nanoplatfrom through spontaneous self-assembly driven by multiple non-covalent interactions, including electrostatic interactions, hydrogen bonding, and hydrophobic forces (Fig. 1A). Within this nanosystem, Dex functions as an anti-inflammatory component to suppress excessive inflammatory signaling, whereas Arg serves as the physiological substrate for endothelial nitric oxide synthase (eNOS)-mediated NO biocatalysis. The combination of these two bioactive molecules was designed to simultaneously attenuate inflammatory activation and restore endogenous NO-associated catalytic regulation, thereby enabling biocatalytic remodeling of the pathological cochlear microenvironment.

Following local administration via tympanic bulla injection, AD was designed to remain within the middle ear cavity and gradually diffuse across the round window membrane (RWM), facilitating local delivery into the inner ear (Fig. 1B). In addition, the positively charged surface characteristics of AD may enhance electrostatic interactions with negatively charged mucosal surfaces of the tympanic cavity and RWM, thereby improving local retention and increasing cochlear exposure. This localized delivery strategy provides a suitable spatial foundation for sustained regulation of the pathological cochlear microenvironment.

The proposed therapeutic mechanism of AD is illustrated in Fig. 1C,D. Noise exposure initially induces oxidative stress, followed by activation of the NF- κ B inflammatory pathway, macrophage polarization imbalance, and disruption of eNOS/iNOS-associated NO regulation. These interconnected pathological alterations collectively contribute to inflammatory and vascular dysfunction within the cochlea. By integrating Dex-mediated inhibition of the ROS/NF- κ B inflammatory axis with Arg-mediated restoration of endogenous eNOS-dependent NO biocatalysis, AD was designed to coordinately regulate inflammatory responses and microvascular-associated dysfunction. Consequently, the AD nanoplatfrom was expected to remodel the pathological cochlear microenvironment and protect cochlear sensory cells and ribbon synapses against noise-induced injury.

3.2 Structural Characterization and Functional Validation of the AD Biocatalytic Nanoplatfrom

The AD biocatalytic nanoplatfrom was successfully constructed through a one-step ultrasound-assisted carrier-free self-assembly strategy (Fig. 2A). Guided by the biocatalytic design rationale described above, Dex and Arg were co-assembled through multiple non-covalent interactions, including electrostatic interactions, hydrogen bonding, and hydrophobic forces, generating a stable nanoplatfrom integrating anti-inflammatory activity with endogenous NO biocatalysis. Following centrifugation and lyophilization, dry AD nanopowder was obtained. Notably, this fabrication strategy avoids the use of additional polymeric carriers or chemical conjugation, thereby preserving the intrinsic biological activities of both bioactive components while simplifying the preparation process and improving its translational potential. Molecular docking analysis further supported the spontaneous self-assembly of Arg and Dex by revealing favorable intermolecular interactions between the two molecules (Supporting Information, Figure S1).

The morphology and physicochemical properties of AD nanoparticles prepared at different Arg:Dex feeding ratios were first characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). All formulations exhibited spherical morphology with relatively uniform size distribution and minimal aggregation (Fig. 2B–D). DLS analysis revealed that nanoparticles prepared at Arg:Dex mass ratios of 1:2, 1:1, and 2:1 exhibited average hydrodynamic diameters of approximately 169.9, 229.8, and 310.7 nm, respectively, with corresponding PDI values of 0.03, 0.08, and 0.02 (Fig. 2E,F). Zeta potential measurements further demonstrated that all formulations possessed positively charged surfaces, with average zeta potentials of + 17.18, + 11.71, and + 20.33 mV, respectively (Fig. 2G). Considering the combined effects of particle size, dispersity, and surface charge, the formulation with an Arg:Dex mass ratio of 1:2 was selected as the optimal formulation for subsequent studies owing to its favorable nanoscale dimensions, excellent colloidal stability, and desirable surface physicochemical properties.

The successful co-assembly of Arg and Dex within the carrier-free nanoplatform was further verified by UV–Vis spectroscopy and X-ray photoelectron spectroscopy (XPS). UV–Vis analysis demonstrated that AD retained the characteristic absorption peaks of both Arg and Dex, indicating the coexistence of the two bioactive components within the nanoplatform (Fig. 2H–J). Consistently, XPS spectra revealed characteristic carbon (C), nitrogen (N), and oxygen (O) signals corresponding to the elemental compositions of Arg and Dex (Fig. 2K). Collectively, these findings confirm the successful co-assembly of Arg and Dex into a structurally stable carrier-free biocatalytic nanoplatform.

The functional properties of AD were subsequently evaluated by investigating its release behavior and hemocompatibility. AD exhibited a sustained release profile, with approximately 70% cumulative release over 72 h (Fig. 2L). Notably, the release rate was accelerated in the presence of H_2O_2 compared with PBS alone, indicating ROS-responsive release under oxidative stress-mimicking conditions. The hemocompatibility of AD was further assessed using a red blood cell (RBC) hemolysis assay. No obvious erythrocyte lysis was observed across the tested concentration range ($0.1\text{--}2\text{ mg mL}^{-1}$), and the hemolysis rate remained consistently below 5% (Fig. 2M). Collectively, these results demonstrate that AD possesses favorable physicochemical properties, including nanoscale dimensions, uniform size distribution, positive surface charge, ROS-responsive sustained release behavior, and excellent hemocompatibility. These characteristics provide a robust structural foundation for prolonged cochlear retention and subsequent biocatalytic remodeling of the pathological cochlear microenvironment.

3.3 Prolonged Cochlear Retention and Local Distribution of AD Following Tympanic Bulla Administration

To evaluate the local retention and cochlear delivery behavior of AD following tympanic bulla administration, AD was physically labeled with the near-infrared fluorescent dye Cy7 to generate Cy7-AD. The in vivo distribution of Cy7-AD was longitudinally monitored using an IVIS fluorescence imaging system (Fig. 3A). Compared with the control group, mice receiving Cy7-AD exhibited strong fluorescence signals in the middle ear region at day 1 after administration. Although the fluorescence intensity

gradually decreased over time, detectable signals remained present at day 7 and persisted until day 14, indicating prolonged retention of AD within the middle ear cavity.

Ex vivo fluorescence imaging of isolated cochleae further confirmed the successful delivery of AD into the inner ear. Detectable Cy7 fluorescence signals were observed in cochlear tissues at days 1, 3, 7, and 14 after administration (Fig. 3A). The temporal distribution pattern in cochlear tissues was consistent with the in vivo imaging results, demonstrating sustained cochlear exposure following local administration. Although fluorescence intensity gradually declined during the observation period, measurable signals remained detectable at day 14, suggesting prolonged cochlear availability of AD.

To assess systemic biodistribution following local delivery, major organs, including the heart, liver, spleen, lung, and kidney, were collected for ex vivo fluorescence imaging. No obvious fluorescence accumulation was observed in these organs throughout the experimental period (Fig. 3B), indicating limited systemic dissemination after tympanic bulla administration.

Collectively, these findings demonstrate that local administration enables prolonged middle-ear retention, sustained cochlear distribution, and limited systemic exposure of AD. These favorable delivery characteristics provide a suitable spatial and temporal basis for subsequent regulation of the pathological cochlear microenvironment.

3.4 AD Remodels the Inflammatory Component of the Pathological Cochlear Microenvironment Under Oxidative Stress

To investigate whether AD could regulate the inflammatory component of the pathological microenvironment under oxidative stress conditions, RAW264.7 macrophages were exposed to H_2O_2 and subsequently treated with Arg, Dex, or AD (Fig. 4A). Preliminary CCK-8 assays identified 400 μM H_2O_2 as the optimal concentration for establishing the oxidative stress model, resulting in a reproducible reduction in cell viability without excessive cytotoxicity (Figure S2).

Intracellular ROS levels were initially evaluated using DCFH-DA fluorescence staining. H_2O_2 stimulation markedly increased intracellular ROS accumulation, confirming successful induction of oxidative stress. Although treatment with either Arg or Dex partially reduced ROS accumulation, AD produced a more pronounced decrease in ROS fluorescence intensity compared with either monotherapy, indicating enhanced regulation of oxidative stress-associated inflammatory responses (Fig. 4D,E).

The effects of AD on macrophage phenotypic regulation were subsequently assessed by flow cytometric analysis of CD206 expression. H_2O_2 stimulation significantly decreased the proportion of CD206-positive macrophages, indicating disruption of the anti-inflammatory macrophage phenotype. In contrast, AD treatment markedly restored CD206 expression, suggesting that AD promoted macrophage polarization toward a reparative phenotype under oxidative stress conditions (Fig. 4B,C).

Immunofluorescence staining further confirmed the regulatory effects of AD on macrophage polarization. H_2O_2 exposure increased the expression of the pro-inflammatory marker iNOS while decreasing the expression of the anti-inflammatory marker Arg-1. AD treatment effectively reversed these alterations by suppressing iNOS expression and restoring Arg-1 expression (Fig. 4F–I). Consistently, RT-qPCR analysis demonstrated that oxidative stress upregulated pro-inflammatory genes, including TNF- α , IL-1 β , and iNOS, while downregulating Arg-1, IL-10, and CD206. AD treatment significantly reversed these transcriptional changes and exhibited superior regulatory effects compared with free Arg or Dex alone (Fig. 4K–P).

Collectively, these findings demonstrate that AD suppresses oxidative stress-associated inflammatory activation, reduces ROS accumulation, and restores macrophage phenotypic balance. These results indicate that AD effectively remodels the inflammatory component of the pathological cochlear microenvironment under oxidative stress conditions (Fig. 4J).

3.5 AD Suppresses NF- κ B Signaling to Mediate Inflammatory Microenvironment Remodeling

To further elucidate the molecular mechanisms underlying AD-mediated inflammatory microenvironment remodeling, transcriptomic sequencing was performed using RAW264.7 macrophages from the Control, H_2O_2 , and H_2O_2 + AD groups. Principal component analysis (PCA) demonstrated high reproducibility among biological replicates within each group and clear separation among the three experimental groups (Fig. 5A). Notably, the transcriptomic profile of the H_2O_2 + AD group was distinctly separated from that of the H_2O_2 group and shifted toward the Control group, indicating that AD partially reversed the global transcriptional alterations induced by oxidative stress.

Differential expression analysis revealed extensive transcriptional changes following AD treatment compared with the H_2O_2 group (Fig. 5B). Heatmap analysis further demonstrated that multiple inflammation-associated genes upregulated by H_2O_2 stimulation were markedly downregulated after AD treatment (Fig. 5D). Consistently, GO and KEGG enrichment analyses showed that the differentially expressed genes were mainly associated with inflammatory responses, immune regulation, cytokine-mediated signaling pathways, and oxidative stress-related biological processes (Fig. 5C), suggesting that AD broadly modulated oxidative stress-induced inflammatory transcriptional remodeling.

To further identify the signaling pathways involved in AD-mediated inflammatory regulation, GSEA was performed. The results demonstrated significant enrichment of inflammation-related gene signatures and NF- κ B signaling pathways in H_2O_2 -treated macrophages, whereas these enrichment patterns were markedly attenuated following AD treatment (Fig. 5E,F). These findings suggested that suppression of NF- κ B signaling may represent an important molecular basis for the anti-inflammatory effects of AD.

To validate the transcriptomic findings, key proteins associated with NF- κ B signaling were examined by Western blot analysis. H_2O_2 stimulation markedly decreased I κ B α expression while increasing the levels

of phosphorylated I κ B α (p-I κ B α), p65, and p50, indicating activation of NF- κ B signaling. In contrast, AD treatment restored I κ B α expression and significantly reduced H₂O₂-induced activation of NF- κ B-related proteins (Fig. 5G).

Taken together, these results demonstrate that AD reverses oxidative stress-induced inflammatory transcriptomic remodeling and suppresses NF- κ B pathway activation, providing molecular evidence for AD-mediated remodeling of the inflammatory component of the pathological microenvironment.

3.6 AD Restores Endogenous NO Biocatalysis and Endothelial Functional Phenotypes Under Oxidative Stress

Given the contribution of endothelial dysfunction and impaired NO regulation to the pathological microenvironment associated with NIHL, we next investigated whether AD could restore endogenous NO biocatalytic function under oxidative stress conditions. HUVECs were exposed to H₂O₂ and subsequently treated with Arg, Dex, or AD. Intracellular NO availability was first assessed using the fluorescent probe DAF-FM DA. H₂O₂ stimulation markedly reduced NO fluorescence intensity, indicating disruption of endothelial NO regulatory capacity under oxidative stress. In contrast, AD treatment significantly restored intracellular NO levels and exhibited superior effects compared with either Arg or Dex treatment alone (Fig. 6A,B).

To further evaluate the molecular basis underlying AD-mediated restoration of NO regulation, the expression of eNOS, iNOS, and VEGFA was analyzed. Oxidative stress markedly decreased eNOS and VEGFA expression while increasing iNOS expression, indicating an imbalance in endothelial NO-associated signaling. AD treatment effectively restored eNOS expression, suppressed oxidative stress-induced iNOS upregulation, and enhanced VEGFA expression (Fig. 6K). These findings were further supported by VEGFA immunofluorescence staining and RT-qPCR analysis, which demonstrated significant recovery of VEGFA expression following AD treatment (Fig. 6G,H,J).

The effects of AD on endothelial functional phenotypes were subsequently evaluated using tube formation and scratch wound healing assays. H₂O₂ exposure markedly impaired endothelial functional capacity, resulting in reduced vascular-like network formation and decreased migratory ability. In contrast, AD treatment significantly improved tube formation performance, as evidenced by increased tubular structures and branch nodes (Fig. 6C,D). Similarly, scratch wound assays demonstrated that AD enhanced HUVEC migration and accelerated wound closure compared with the H₂O₂ group (Fig. 6E,F). Consistent with these functional improvements, phalloidin staining showed that AD preserved F-actin organization and improved cytoskeletal integrity under oxidative stress conditions (Fig. 6I).

Collectively, these findings demonstrate that AD restores endogenous NO biocatalytic regulation and improves endothelial functional phenotypes under oxidative stress. These results provide in vitro evidence that AD can regulate the vascular-associated component of the pathological microenvironment through coordinated modulation of NO-associated signaling and endothelial function.

3.7 AD-Mediated Microenvironment Regulation Protects HEI-OC1 Cells Against Oxidative Injury

To evaluate whether AD-mediated pathological microenvironment regulation could protect cochlear sensory cell-like cells against oxidative injury, HEI-OC1 cells were exposed to H_2O_2 and subsequently treated with Arg, Dex, or AD. Cell viability was first evaluated using Calcein-AM/PI live/dead staining. Control cells exhibited strong green fluorescence with minimal red fluorescence, indicating high cell viability. In contrast, H_2O_2 exposure markedly reduced viable cells and increased cell death. Treatment with AD significantly increased the proportion of viable cells and attenuated oxidative injury compared with the H_2O_2 group. Moreover, the protective effect of AD was superior to that observed with either Arg or Dex monotherapy (Fig. 7A,B).

To further evaluate the effects of AD on cellular structural preservation, F-actin organization was examined by phalloidin staining. HEI-OC1 cells in the Control group exhibited well-spread morphology with organized actin filaments. Following H_2O_2 stimulation, cells displayed obvious shrinkage, reduced spreading, and disrupted cytoskeletal organization. In contrast, AD treatment effectively preserved cellular morphology, restored F-actin organization, and promoted cell spreading under oxidative stress conditions (Fig. 7C).

The cytocompatibility of AD was further assessed using the CCK-8 assay. AD showed negligible cytotoxicity across the tested concentration range, demonstrating favorable biocompatibility for subsequent biological evaluation (Fig. 7D).

Collectively, these findings demonstrate that AD protects HEI-OC1 cells against oxidative injury by improving cell survival, preserving cytoskeletal organization, and exhibiting excellent cytocompatibility. These results further support that AD-mediated pathological microenvironment regulation contributes to cochlear sensory cell protection.

3.8 Transcriptomic Analysis Reveals Hedgehog-Associated Cytoprotective Responses Induced by AD

To further investigate the molecular basis underlying AD-mediated cytoprotection in HEI-OC1 cells, transcriptomic sequencing was performed using cells from the Control, H_2O_2 , and H_2O_2 + AD groups. PCA demonstrated clear separation among the three experimental groups, while the transcriptomic profile of the H_2O_2 + AD group shifted toward that of the Control group compared with the H_2O_2 group, indicating partial reversal of oxidative stress-induced transcriptional alterations by AD (Fig. 8A).

Differential expression analysis revealed extensive transcriptional remodeling following AD treatment under oxidative stress conditions (Fig. 8B). GO and KEGG enrichment analyses demonstrated that the differentially expressed genes were mainly associated with oxidative stress responses, cellular homeostasis, extracellular matrix organization, and cytoprotective biological processes (Fig. 8C). GSEA

further showed that ROS-associated signaling pathways were significantly enriched following H₂O₂ stimulation, whereas these alterations were attenuated after AD treatment (Fig. 8D).

Interestingly, Hedgehog-related signaling was positively enriched in the AD-treated group (Fig. 8E). Heatmap analysis further revealed differential regulation of genes associated with cellular homeostasis and cytoprotective responses following AD treatment (Fig. 8F,G). To validate the transcriptomic findings, HO-1 expression and Hedgehog-associated molecules were further examined. H₂O₂ stimulation increased HO-1 expression, whereas AD treatment reduced HO-1 fluorescence intensity, indicating attenuation of oxidative stress responses (Fig. 8H,I). In addition, AD treatment increased the expression of Gli1 and Ptch1, consistent with activation of Hedgehog-associated protective responses (Fig. 8J–M).

Collectively, these findings demonstrate that AD alleviates oxidative stress-associated damage in HEI-OC1 cells and induces cytoprotective transcriptional responses involving Hedgehog-associated signaling. However, because pharmacological inhibition or genetic manipulation of Hedgehog signaling was not performed, these results indicate an associated protective response rather than establishing a definitive causal role for Hedgehog signaling.

3.9 AD Preserves Auditory Function and Cochlear Structures in NIHL Mice

To evaluate the therapeutic efficacy of AD *in vivo*, a mouse model of NIHL was established. Mice were randomly assigned to five experimental groups: Control, Noise, Noise + Arg, Noise + Dex, and Noise + AD. The corresponding treatments were administered via tympanic bulla injection one day before noise exposure. Auditory function was evaluated by ABR measurements before noise exposure and at days 3, 7, and 14 after acoustic stimulation (Fig. 9A). Complete ABR threshold data across all tested frequencies are provided in Supporting Information (Figure S3).

Baseline ABR measurements showed no significant differences among the experimental groups before noise exposure, confirming comparable auditory function prior to acoustic injury (Fig. 9B,C). Following exposure to broadband noise (110 dB SPL, 2 h), mice in the Noise group exhibited substantial increases in ABR thresholds at 8, 16, and 32 kHz, confirming successful establishment of the NIHL model. Although treatment with Arg or Dex alone partially reduced noise-induced auditory impairment, AD treatment produced a more pronounced attenuation of ABR threshold shifts throughout the observation period. The protective effects of AD were particularly evident at 16 and 32 kHz, frequencies highly susceptible to noise-induced cochlear injury (Fig. 9D–F).

To determine whether the functional improvement was associated with preservation of cochlear sensory structures, cochlear whole-mount immunostaining was performed. Myosin VIIa and phalloidin staining revealed substantial outer hair cell loss after noise exposure, particularly in the middle and basal cochlear turns. In contrast, AD treatment markedly preserved the orderly arrangement of outer hair cells and reduced noise-induced hair cell loss (Fig. 9G–I). Quantitative analysis further confirmed significantly

increased outer hair cell survival in the middle and basal turns of AD-treated mice compared with the Noise group, consistent with the improved high-frequency hearing preservation observed in ABR measurements (Fig. 9K–M).

Ribbon synapse integrity was further evaluated by double immunofluorescence staining for CtBP2 and GluR2. Noise exposure resulted in a marked reduction in CtBP2/GluR2 co-localized puncta within inner hair cells, indicating degeneration of ribbon synapses after acoustic injury. In contrast, AD treatment significantly preserved paired CtBP2/GluR2 puncta per inner hair cell, suggesting effective protection of functional synaptic connections following noise exposure (Fig. 9J,N).

Collectively, these findings demonstrate that AD effectively protects cochlear functional units against noise-induced injury by preserving auditory function, outer hair cells, and ribbon synapse integrity. These results provide *in vivo* evidence supporting the therapeutic efficacy of AD as a locally administered intervention for NIHL.

3.10 AD Remodels the Cochlear Inflammatory–Vascular Microenvironment In Vivo

To further validate whether AD could remodel the pathological cochlear microenvironment *in vivo*, cochlear inflammatory responses, histopathological alterations, and NO-associated vascular signaling were systematically evaluated in cochlear tissues following noise exposure (Fig. 10).

RT-qPCR analysis demonstrated that acoustic overexposure markedly increased the expression of pro-inflammatory mediators, including TNF- α , IL-1 β , and iNOS, while decreasing the expression of anti-inflammatory markers, including Arg-1, CD206, and IL-10 (Fig. 10A–F). These alterations indicated persistent inflammatory activation and disruption of immune homeostasis within the cochlea after noise exposure. Compared with the Noise group, AD treatment significantly reduced the expression of TNF- α , IL-1 β , and iNOS while restoring Arg-1, CD206, and IL-10 expression. Moreover, AD exhibited stronger regulatory effects than either Arg or Dex monotherapy, suggesting that the self-assembled nanoplatform achieved enhanced immunomodulatory activity through the coordinated functions of its two bioactive components.

Histopathological examination by H&E staining further revealed that noise exposure caused apparent cochlear structural damage, characterized by disruption of tissue architecture and degeneration of cochlear structures (Fig. 10G). In contrast, AD-treated mice exhibited improved preservation of cochlear morphology and reduced histopathological alterations following noise exposure.

To investigate whether AD could regulate the vascular-associated component of the pathological cochlear microenvironment, immunohistochemical staining was performed to evaluate eNOS and iNOS expression in cochlear tissues (Fig. 10H–K). Noise exposure resulted in decreased eNOS expression accompanied by increased iNOS expression, indicating disruption of eNOS/iNOS-associated NO regulation within the cochlea. AD treatment effectively restored eNOS expression and suppressed noise-

induced iNOS upregulation. Quantitative analysis further confirmed that AD corrected the noise-induced imbalance between eNOS and iNOS, supporting restoration of endogenous NO-associated regulatory capacity in the cochlea.

Collectively, these findings demonstrate that AD simultaneously regulates cochlear inflammatory activation and vascular-associated NO signaling *in vivo*. Through coordinated modulation of inflammatory responses and endogenous NO biocatalytic regulation, AD effectively remodels the pathological cochlear microenvironment, providing a mechanistic basis for its protective effects against noise-induced cochlear injury.

4. Discussion

Noise-induced hearing loss (NIHL) has traditionally been considered a consequence of the combined effects of mechanical injury, disruption of ionic homeostasis, and secondary oxidative stress. Among these pathological events, excessive reactive oxygen species (ROS) accumulation is widely recognized as a major driver of cochlear sensory cell injury and progressive auditory dysfunction, making antioxidant intervention a longstanding therapeutic strategy in NIHL research. Previous experimental studies and clinical trials have demonstrated that antioxidants, including N-acetylcysteine (NAC), can partially alleviate noise-induced auditory threshold shifts. Nevertheless, their overall therapeutic efficacy remains limited, and clinical outcomes have shown considerable variability.^[27–30] These observations indicate that NIHL progression cannot be fully attributed to ROS-mediated damage alone but rather involves a complex pathological cascade initiated and sustained by oxidative stress. Increasing evidence has demonstrated that acoustic overexposure is accompanied by immune activation, macrophage recruitment and polarization changes, excessive inflammatory cytokine production, stria vascularis dysfunction, and impairment of cochlear microcirculation.^[30–32] Collectively, these findings have expanded our understanding of NIHL as a multifactorial disorder involving interconnected pathological networks. However, most previous studies have investigated these alterations as independent events, primarily focusing on individual pathological processes such as oxidative stress, inflammation, or vascular dysfunction.^[33, 34] Therefore, the coordinated interactions among these processes and the development of therapeutic strategies capable of simultaneously restoring the pathological cochlear microenvironment remain insufficiently explored.

Accordingly, we propose pathological cochlear microenvironment remodeling as a conceptual framework for understanding NIHL progression and therapeutic intervention. Rather than representing isolated pathological events, inflammatory activation and vascular dysfunction constitute a highly interconnected regulatory network that continuously amplifies oxidative stress-induced cochlear injury. Persistent ROS accumulation activates NF- κ B signaling, promotes pro-inflammatory macrophage polarization, and induces excessive production of inflammatory mediators, thereby further impairing endothelial function and disrupting vascular-associated homeostasis. Conversely, vascular dysfunction characterized by impaired endothelial NO availability and abnormal angiogenic regulation may reinforce inflammatory activation and compromise the intrinsic repair capacity of cochlear tissues. Therefore,

therapeutic strategies capable of simultaneously restoring inflammatory balance and vascular-associated regulatory functions may provide advantages over approaches targeting oxidative stress alone. In this study, we developed a carrier-free Arg–Dex (AD) self-assembled biocatalytic nanoplatform designed to coordinately regulate these interconnected pathological processes. By integrating Dex-mediated inflammatory regulation with Arg-mediated endogenous NO biocatalysis, AD enables coordinated remodeling of the pathological cochlear microenvironment rather than single-pathway modulation.

A key finding of this study is that AD effectively remodels the inflammatory component of the pathological cochlear microenvironment. Dex, a well-established glucocorticoid with potent anti-inflammatory activity, has been widely investigated for the treatment of inflammatory disorders; however, its therapeutic application in inner-ear diseases remains limited by rapid clearance and insufficient local availability. By integrating Dex into a carrier-free self-assembled nanoplatform with Arg, we established a localized therapeutic system capable of sustained bioactive regulation within the cochlear pathological environment. Our *in vitro* findings demonstrated that AD reduced ROS accumulation, suppressed iNOS expression, restored Arg-1 and CD206 expression, and promoted macrophage phenotypic transition toward an anti-inflammatory state under oxidative stress conditions. Transcriptomic analysis further revealed that AD reversed oxidative stress-induced inflammatory transcriptional remodeling and attenuated NF- κ B pathway activation, which was further validated by restoration of I κ B α expression and inhibition of p-I κ B α , p65, and p50 activation. These findings suggest that AD functions beyond conventional inflammatory suppression by restoring macrophage inflammatory balance and remodeling the inflammatory state of the pathological microenvironment. Such a microenvironment-oriented immunomodulatory strategy may provide particular advantages for NIHL, in which persistent inflammatory activation contributes to progressive cochlear degeneration.

In addition to inflammatory regulation, AD also addresses vascular-associated dysfunction, another critical but relatively underexplored component of NIHL pathology. The cochlea relies on precise vascular support to maintain metabolic demands, and impairment of endothelial function within the stria vascularis may compromise local homeostasis and increase the vulnerability of sensory cells to acoustic stress. NO signaling plays an essential role in regulating endothelial function, angiogenic responses, and vascular-associated physiological processes. However, oxidative stress can disrupt eNOS activity while inducing excessive iNOS activation, leading to impaired NO regulatory capacity and endothelial dysfunction. In this study, Arg was incorporated into the AD nanoplatform as a physiological substrate supporting eNOS-mediated endogenous NO biocatalysis. Our results demonstrated that AD restored NO availability, corrected the imbalance between eNOS and iNOS expression, enhanced VEGFA expression, and improved endothelial functional phenotypes, including tube formation and migration. Importantly, these effects were more pronounced than those achieved with Arg monotherapy, suggesting that integration with Dex and nanoscale self-assembly enables coordinated regulation of NO-associated vascular signaling. Through restoration of endogenous NO biocatalytic regulation, AD contributes to remodeling of the vascular-associated component of the pathological cochlear microenvironment, thereby complementing its inflammatory regulatory effects.

The superior therapeutic efficacy of AD compared with individual components highlights the importance of coordinated regulation of multiple interconnected pathological processes. Conventional pharmacological approaches for NIHL often focus on individual targets, such as ROS scavenging or inflammatory suppression; however, these strategies may be insufficient to overcome the complex pathological network established after acoustic injury. By integrating Arg and Dex into a single carrier-free self-assembled nanoplateform, AD simultaneously regulates inflammatory activation and vascular-associated dysfunction, two closely interconnected components of the pathological cochlear microenvironment. This integrated design provides several advantages. First, the carrier-free architecture avoids the use of additional delivery materials while preserving the intrinsic biological activities of both bioactive components. Second, the self-assembly strategy enables localized cochlear delivery and sustained availability of therapeutic components following administration. Third, the dual-functional design allows complementary regulation of inflammatory signaling and endogenous NO biocatalytic processes within the same therapeutic platform. Therefore, AD represents a transition from conventional single-pathway intervention toward biocatalytic microenvironment remodeling, providing a potential therapeutic paradigm for complex diseases characterized by interconnected pathological dysregulation.

Beyond inflammatory and vascular-associated regulation, AD also provided direct protection to cochlear sensory cell-like cells under oxidative stress conditions. Oxidative injury to sensory cells represents a critical event in NIHL progression, and preservation of cellular integrity is essential for maintaining cochlear function. In HEI-OC1 cells, AD significantly improved cell survival, preserved cytoskeletal organization, and reduced oxidative stress-associated cellular damage. Transcriptomic analysis further indicated that AD-induced cytoprotection was accompanied by activation of endogenous stress-responsive and protective transcriptional programs, including Hedgehog-associated signaling responses. Hedgehog signaling has been implicated in cellular stress adaptation, antioxidant defense, and tissue protection in various biological contexts. Consistent with the transcriptomic findings, AD treatment increased the expression of Gli1 and Ptch1 while attenuating oxidative stress-associated responses. Although the precise role of Hedgehog signaling in AD-mediated cochlear protection requires further investigation, these observations suggest that AD may enhance intrinsic cytoprotective responses beyond its regulation of inflammatory and vascular-associated pathways. Importantly, the present findings identify Hedgehog-related signaling as an associated protective response rather than a definitive causal mechanism underlying AD-induced cellular protection.

From a translational perspective, the carrier-free design of AD provides several advantages compared with conventional nanoparticle-based delivery systems. Many nanocarriers require complex fabrication procedures, rely on additional excipients, and may introduce concerns related to biological compatibility or interference with the activity of incorporated therapeutics. In contrast, AD is constructed through spontaneous self-assembly of two clinically established bioactive molecules without additional polymeric carriers or chemical conjugation. This simplified fabrication strategy preserves the intrinsic biological functions of Arg and Dex while generating a stable nanoplateform capable of localized therapeutic regulation. Furthermore, tympanic bulla administration enabled prolonged retention within the middle ear, sustained cochlear distribution, and limited systemic exposure, thereby overcoming a

major challenge associated with inner-ear drug delivery. Importantly, these delivery characteristics provide a favorable spatial and temporal framework for sustained biocatalytic regulation of the pathological cochlear microenvironment. Collectively, the carrier-free AD nanoplatfrom represents a promising and potentially translatable strategy for localized treatment of NIHL and other inner-ear disorders involving complex pathological microenvironment dysregulation.

Several limitations should be acknowledged in this study. First, although HUVECs were employed to evaluate endothelial responses, further investigations using primary cochlear endothelial cells, advanced vascular models, or in vivo microvascular imaging approaches are required to directly validate the effects of AD on cochlear vascular function. Second, although Hedgehog-associated signaling responses were identified through transcriptomic analysis and further validated experimentally, the causal contribution of this pathway to AD-mediated cytoprotection requires additional investigation using pathway-specific inhibition or genetic manipulation approaches. Third, although AD exhibited favorable biocompatibility and safety profiles within the current experimental framework, further studies involving long-term toxicity evaluation, pharmacokinetic characterization, and clinical translation assessment are warranted.

Despite these limitations, this study establishes a biocatalytic microenvironment remodeling strategy for NIHL treatment by integrating inflammatory regulation and endogenous NO biocatalytic control within a single carrier-free nanoplatfrom. Rather than targeting a single pathological event, the AD platform enables coordinated regulation of interconnected inflammatory and vascular-associated disturbances within the pathological cochlear microenvironment, thereby providing functional and structural protection against noise-induced injury. More broadly, this work highlights the potential of biocatalytic nanomaterials as a therapeutic paradigm for complex diseases characterized by coupled pathological networks and provides a framework for designing multifunctional nanoplatfroms capable of restoring disease-associated microenvironments.

5. Conclusion

In summary, this study developed a carrier-free Arg–Dex (AD) self-assembled biocatalytic nanoplatfrom for localized inner-ear delivery and systematically evaluated its therapeutic effects against noise-induced hearing loss (NIHL). The AD nanoplatfrom exhibited favorable physicochemical characteristics, excellent biocompatibility, prolonged cochlear retention, and efficient inner-ear distribution following tympanic bulla administration. Functionally, AD effectively reduced oxidative stress-associated inflammatory activation, restored endogenous NO regulatory capacity, protected cochlear sensory cells from injury, and ultimately preserved auditory function, outer hair cell survival, and ribbon synapse integrity in a mouse model of NIHL.

Mechanistically, the therapeutic effects of AD were achieved through the coordinated functions of its two bioactive components. Dex-mediated suppression of the ROS/NF- κ B inflammatory axis and Arg-mediated restoration of eNOS-associated endogenous NO biocatalysis jointly regulated inflammatory

activation and vascular-associated dysfunction following acoustic trauma. These findings indicate that NIHL progression is not driven solely by oxidative stress but involves the establishment of a pathological cochlear microenvironment characterized by interconnected inflammatory and vascular disturbances.

Importantly, this study establishes a biocatalytic microenvironment remodeling strategy for NIHL treatment. Rather than targeting a single pathological pathway, the carrier-free AD nanoplatfom enables coordinated regulation of multiple interconnected pathological processes within the cochlear microenvironment, thereby providing functional and structural protection against noise-induced injury. Beyond advancing our understanding of NIHL pathogenesis, this work highlights the potential of biocatalytic nanomaterials as a therapeutic paradigm for complex diseases associated with pathological microenvironment dysregulation and provides a foundation for developing multifunctional nanoplatfoms for inner-ear therapy.

Declarations

Competing Interests

The authors declare that they have no competing interests.

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Author Contribution

R and X contributed to the study design, methodology, conceptualization, performed the experiments, analyzed the data, and wrote the original draft of the manuscript. C, B, W, M, J, S, H, Z, and X contributed to the preliminary data collection and background research. Q and Q contributed to the study design, methodology, supervision, and revision of the manuscript. Z and S supervised the research, revised the manuscript, provided resources, and secured funding for the project. The manuscript was reviewed and approved by all the authors.

Data Availability

Data will be available from the corresponding author upon reasonable request.

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Figures

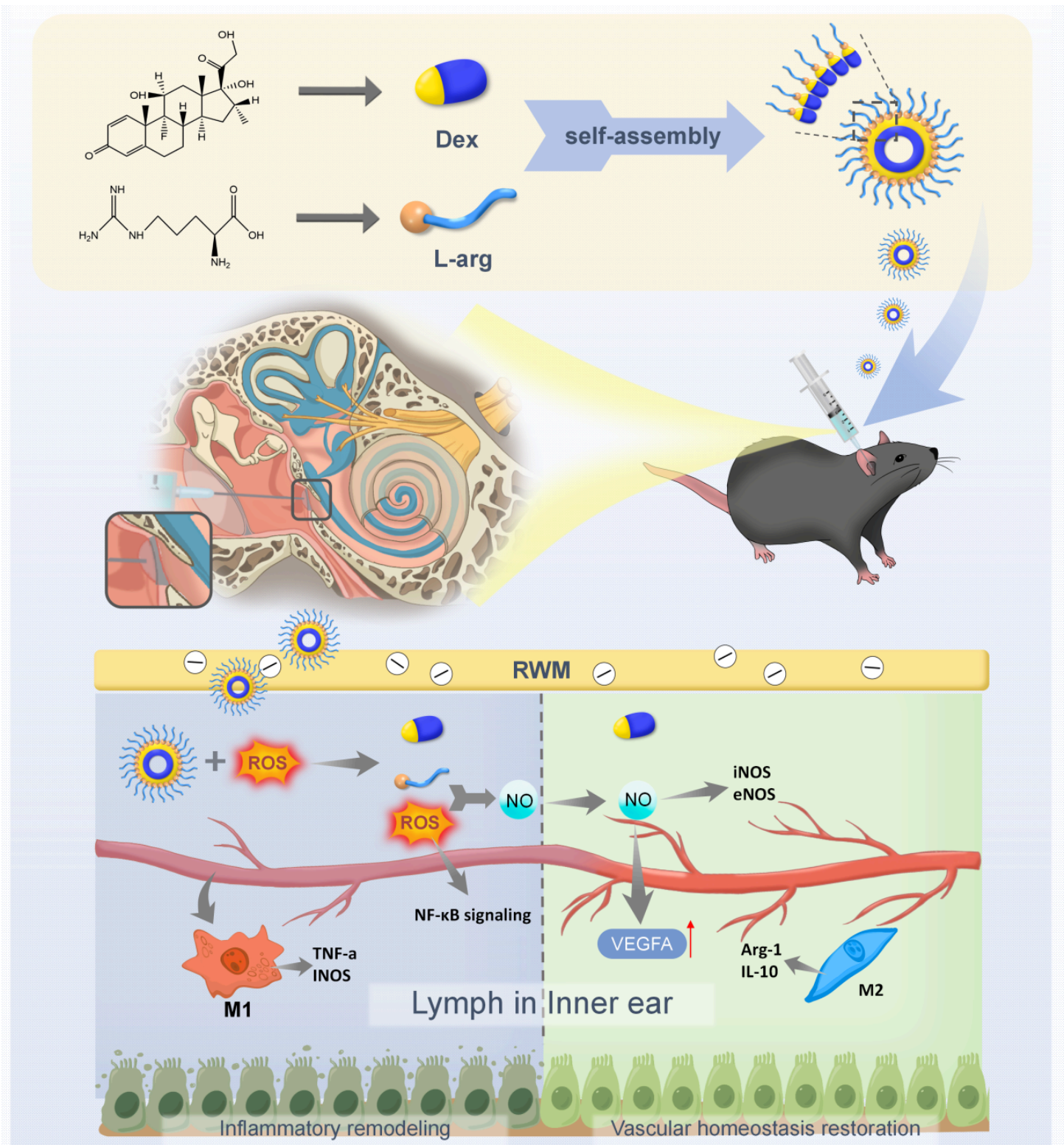


Figure 1

Design rationale and biocatalytic therapeutic strategy of the Arg-Dex (AD) self-assembled nanoplatform for noise-induced hearing loss (NIHL). (A) Schematic illustration of the carrier-free self-assembly of L-arginine (Arg) and dexamethasone (Dex) into the AD nanoplatform through electrostatic interactions, hydrogen bonding, and hydrophobic interactions. Dex functions as an anti-inflammatory component, whereas Arg serves as the physiological substrate for endothelial nitric oxide synthase

(eNOS)-mediated nitric oxide (NO) biocatalysis.(B) Schematic representation of local cochlear delivery following tympanic bulla administration, illustrating middle-ear retention, diffusion across the round window membrane (RWM), and cochlear distribution of the AD nanoplatfrom.(C) Schematic illustration of the pathological cascade underlying NIHL. Acoustic overexposure induces reactive oxygen species (ROS) accumulation, NF- κ B-mediated inflammatory activation, macrophage polarization imbalance, and disruption of eNOS/iNOS-associated NO regulation, contributing to the establishment of a pathological cochlear microenvironment characterized by inflammatory and vascular dysfunction.(D) Proposed biocatalytic therapeutic mechanism of the AD nanoplatfrom. By integrating Dex-mediated suppression of inflammatory signaling with Arg-mediated restoration of endogenous eNOS-dependent NO biocatalysis, AD enables remodeling of the pathological cochlear microenvironment and protection of cochlear sensory hair cells and ribbon synapses against noise-induced injury.

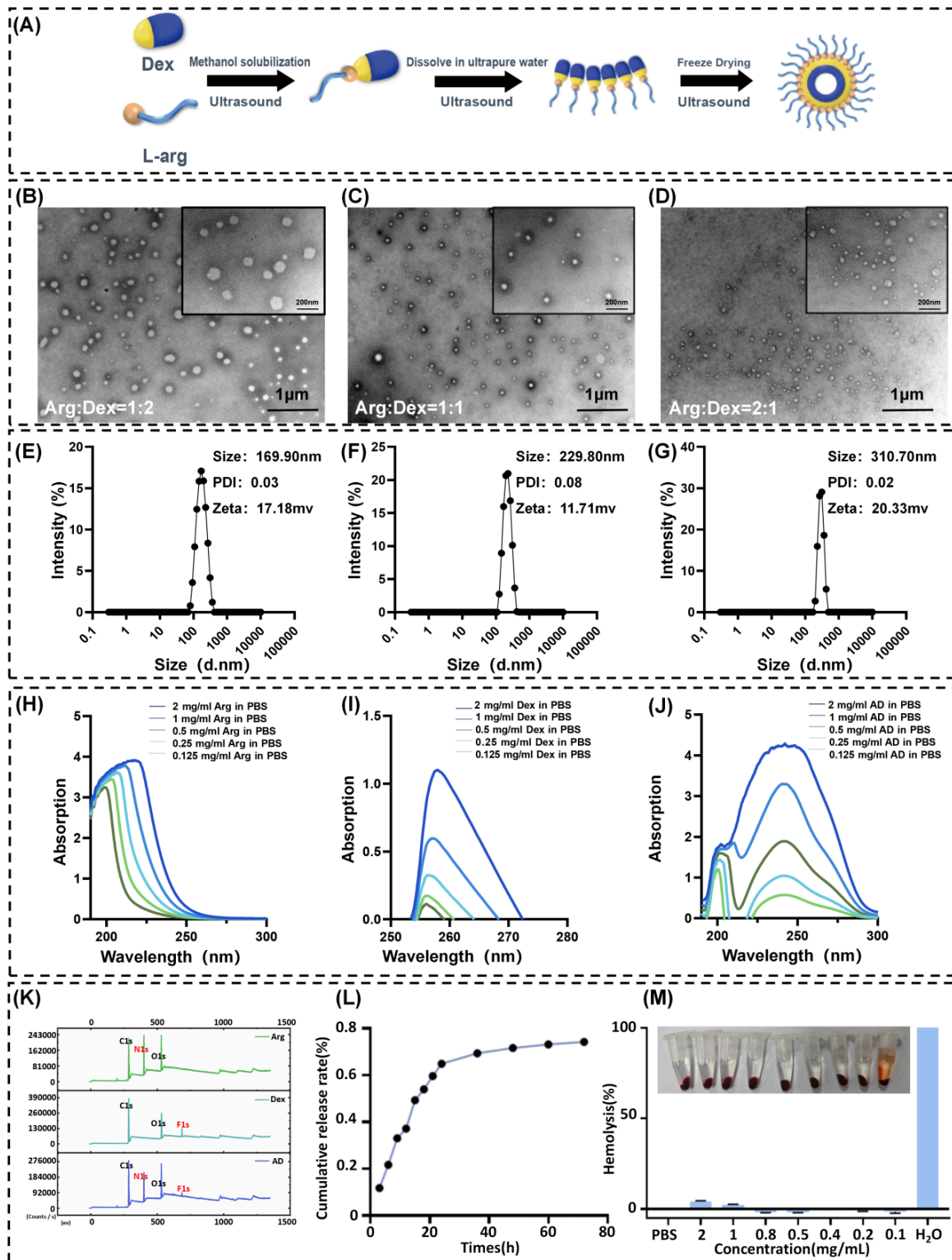


Figure 2

Structural and physicochemical characterization of the AD biocatalytic nanoplatform. (A) Schematic illustration of the ultrasound-assisted carrier-free self-assembly process for constructing the AD nanoplatform. (B–D) Representative transmission electron microscopy (TEM) images of AD nanoplatforms prepared at Arg:Dex feeding ratios of 1:2, 1:1, and 2:1, respectively. (E) Hydrodynamic size distributions of different formulations determined by dynamic light scattering (DLS). (F) Polydispersity

index (PDI) of different formulations. (G) Zeta potential measurements of different formulations. (H–J) UV–Vis absorption spectra of Arg, Dex, and AD. (K) X-ray photoelectron spectroscopy (XPS) characterization of the AD nanoplatform. (L) In vitro cumulative release profiles of AD in PBS and oxidative stress-mimicking PBS containing H₂O₂. (M) Hemolysis assay evaluating the hemocompatibility of the AD nanoplatform at different concentrations. Data are presented as mean ± SD (n = 3 independent experiments).

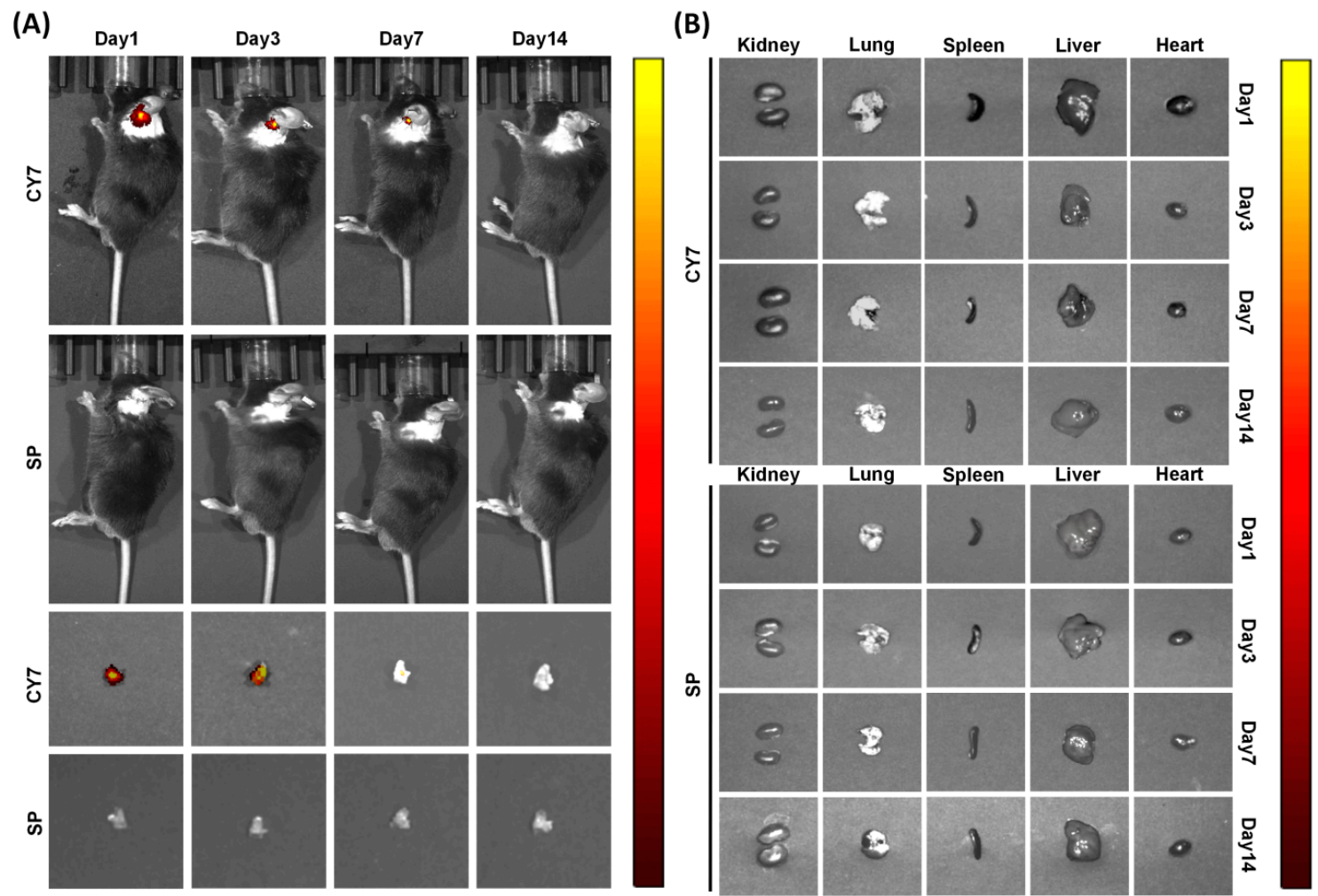


Figure 3

Prolonged cochlear retention and local distribution of the AD biocatalytic nanoplatform following tympanic bulla administration. Representative in vivo fluorescence images and ex vivo cochlear fluorescence images of Cy7-labeled AD (Cy7-AD) acquired at 1, 3, 7, and 14 days after tympanic bulla administration. (B) Ex vivo fluorescence images of major organs (heart, liver, spleen, lung, and kidney) collected at the indicated time points after local administration. Representative images from three independent animals (n = 3).

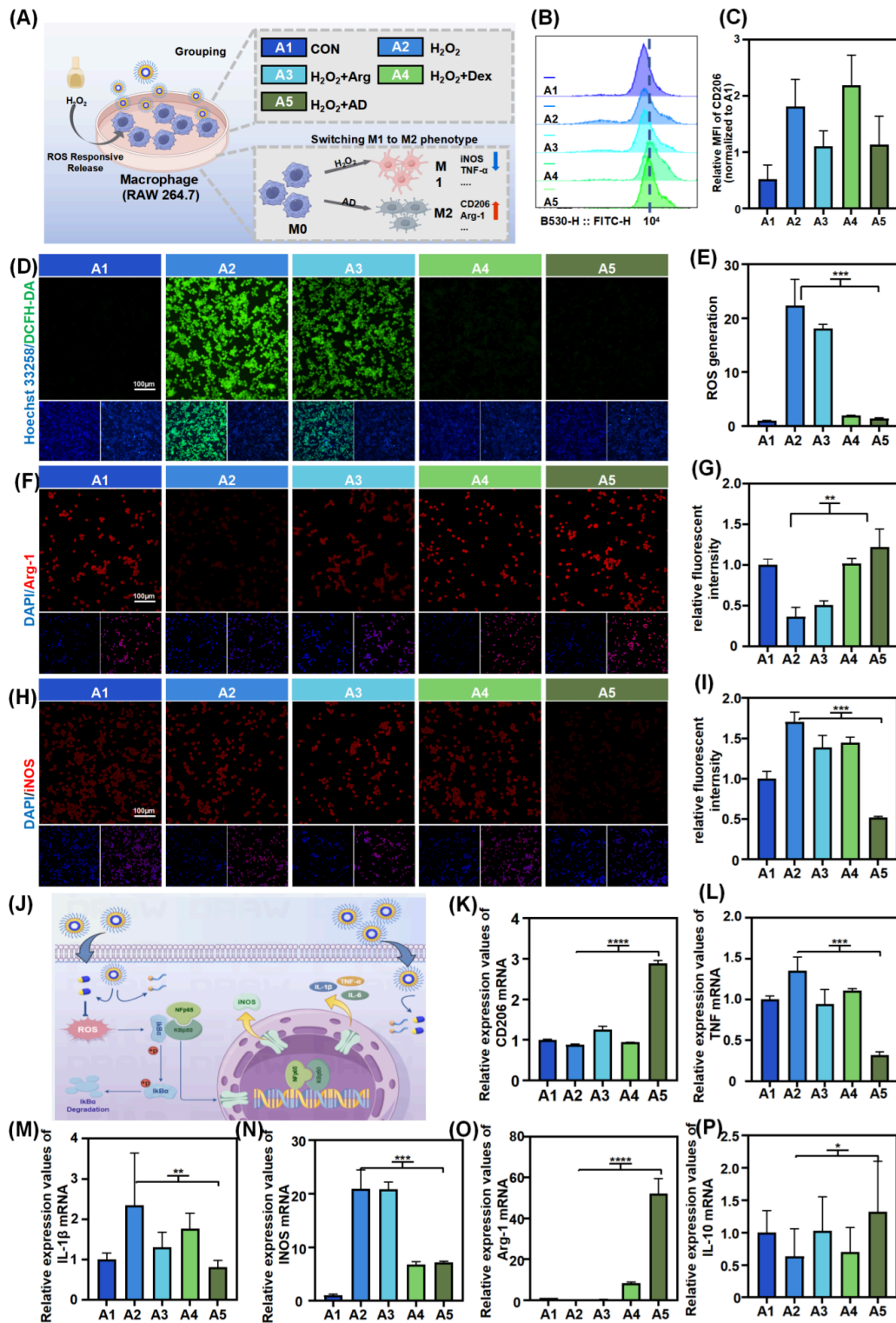


Figure 4

AD remodels the inflammatory component of the pathological microenvironment under oxidative stress conditions. (A) Experimental design for oxidative stress induction and treatment of RAW264.7 macrophages. (B) Representative flow cytometry plots of CD206-positive macrophages. (C) Quantification of CD206-positive macrophages. (D) Representative DCFH-DA fluorescence images showing intracellular ROS levels. (E) Quantification of ROS fluorescence intensity. (F) Representative

immunofluorescence staining images of iNOS expression. (G) Quantification of iNOS fluorescence intensity. (H) Representative immunofluorescence staining images of Arg-1 expression. (I) Quantification of Arg-1 fluorescence intensity. (J) Schematic illustration of AD-mediated regulation of macrophage polarization under oxidative stress. (K–P) RT-qPCR analysis of TNF- α , IL-1 β , iNOS, Arg-1, IL-10, and CD206 expression. Data are presented as mean $\pm$ SD (n = 3 independent experiments). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with the H₂O₂ group. Scale bars, 100 μ m.

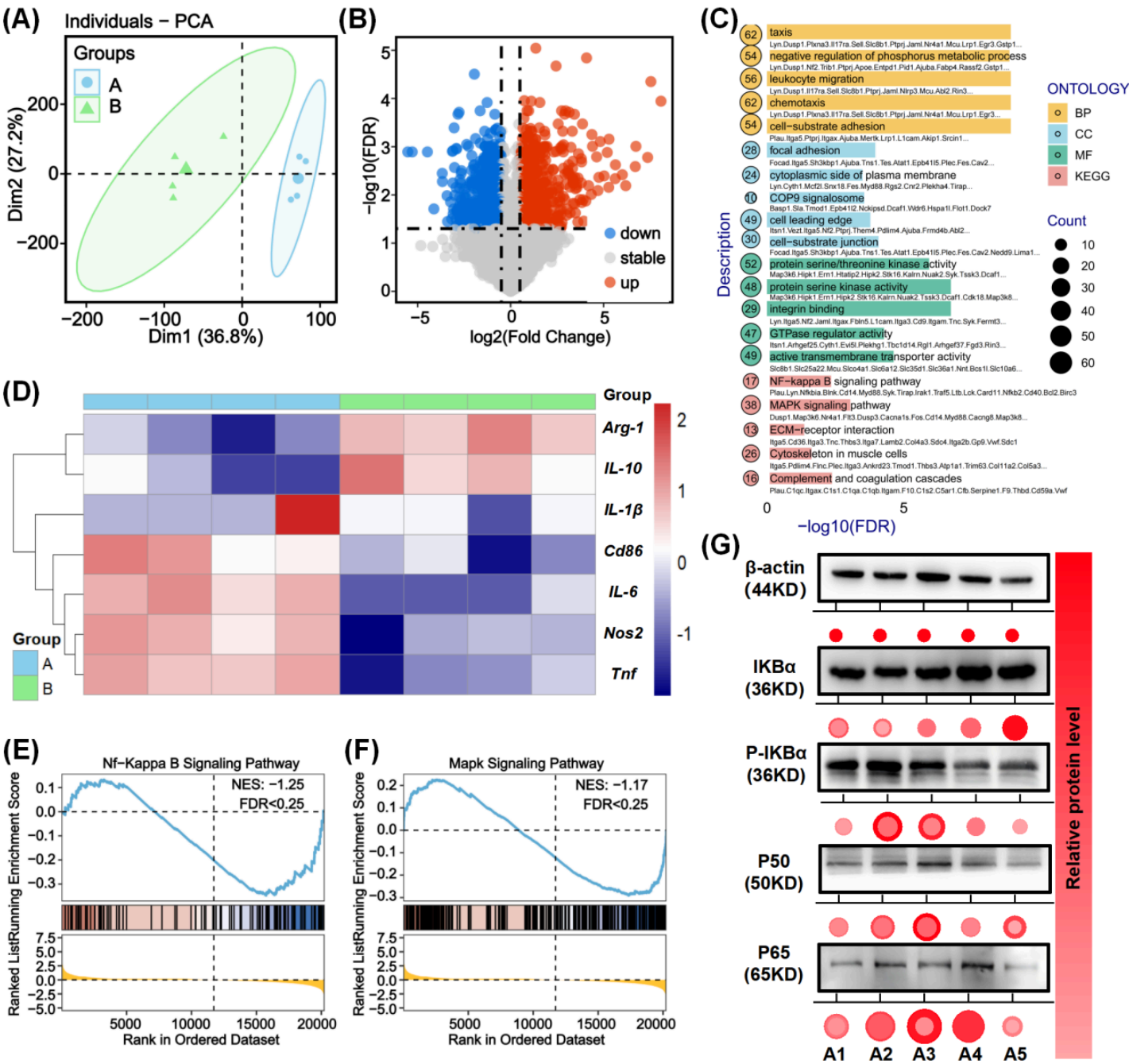


Figure 5

Transcriptomic and molecular analysis of AD-mediated inflammatory regulation under oxidative stress.

(A) Principal component analysis (PCA) of transcriptomic profiles from different treatment groups. (B) Volcano plot showing differentially expressed genes. (C) GO and KEGG enrichment analyses of differentially expressed genes. (D) Heatmap of representative inflammation-related genes. (E–F) Gene set enrichment analysis (GSEA) of inflammation-related pathways and NF- κ B signaling pathway. (G) Western blot analysis of I κ B α , p-I κ B α , p65, and p50 expression and corresponding quantification. Data are presented as mean $\pm$ SD (n = 3 independent experiments). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with the H₂O₂ group.

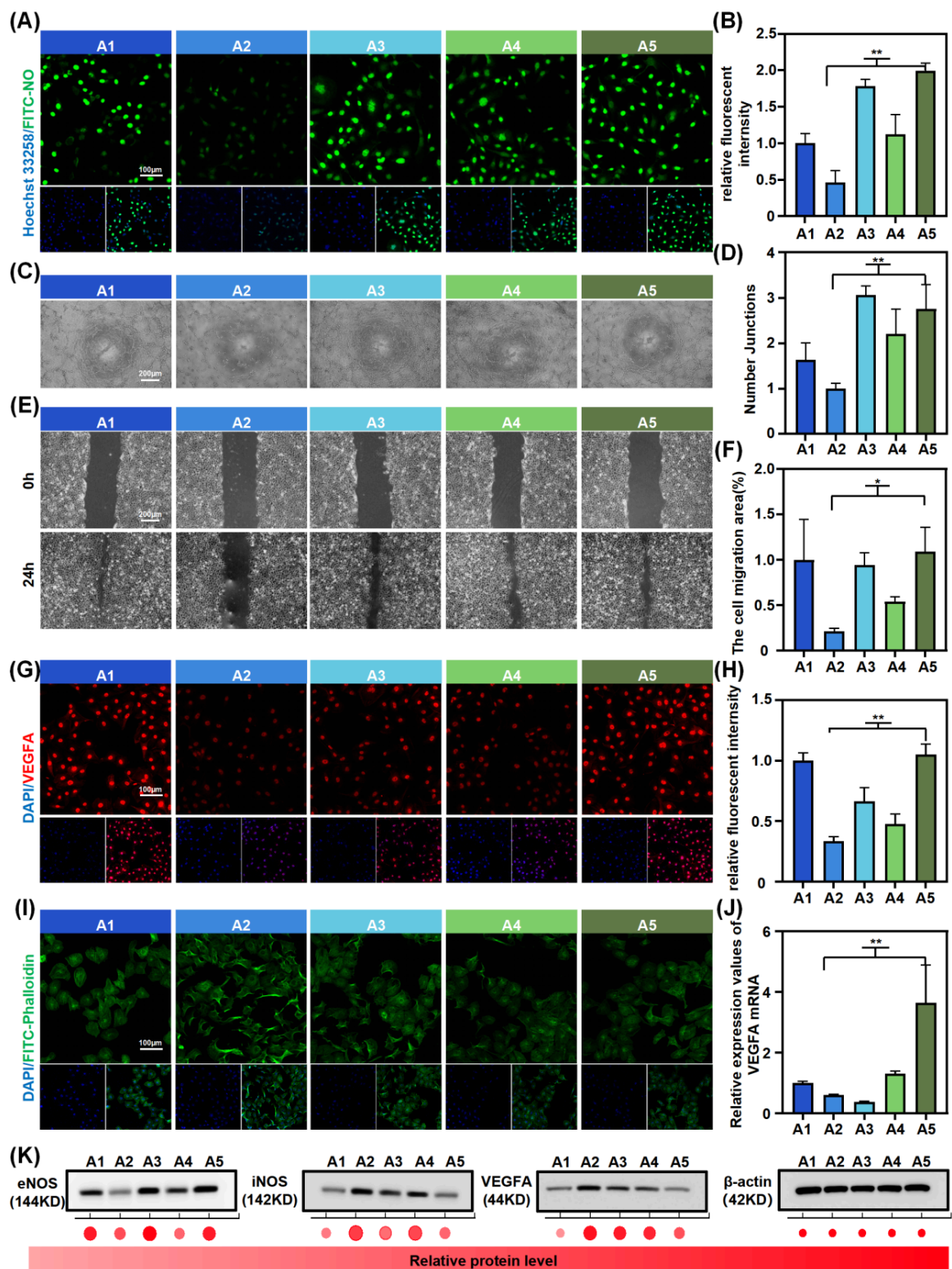


Figure 6

AD restores endogenous NO biocatalysis and endothelial functional phenotypes under oxidative stress.

(A) Representative DAF-FM DA fluorescence images showing intracellular nitric oxide (NO) levels. (B) Quantification of NO fluorescence intensity. (C–D) Representative images and quantification of tube formation assays. (E–F) Representative images and quantification of scratch wound healing assays. (G–H) Representative immunofluorescence images and quantification of VEGFA expression. (I)

Representative phalloidin staining images showing cytoskeletal organization. (J) RT-qPCR analysis of VEGFA and HIF-1 α expression. (K) Western blot analysis of eNOS, iNOS, and VEGFA expression. Data are presented as mean $\pm$ SD (n = 3 independent experiments). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with the H₂O₂ group. Scale bars, 100 μ m and 200 μ m.

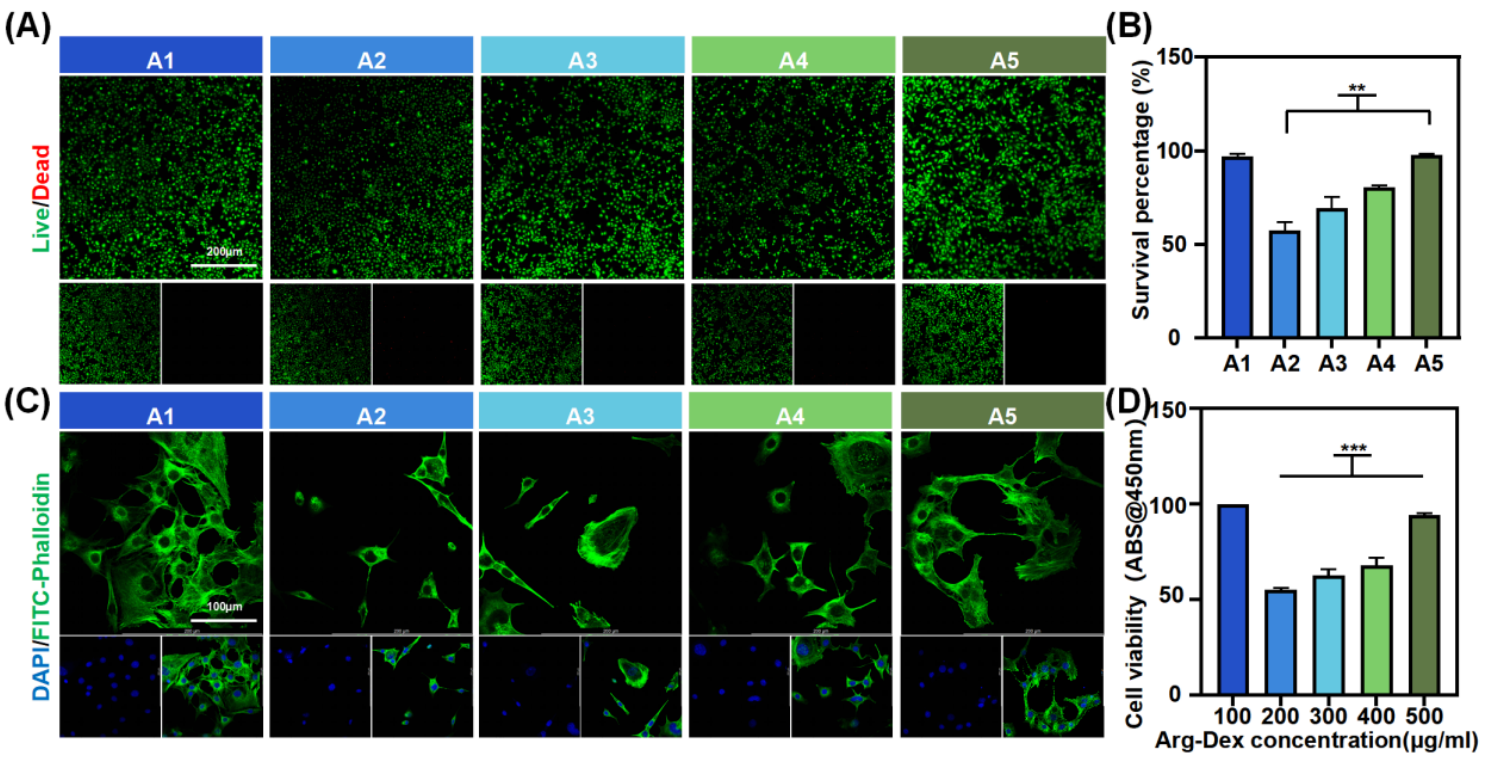


Figure 7

AD protects cochlear sensory cell-like cells against oxidative injury. (A) Representative Calcein-AM/PI live/dead staining images of HEI-OC1 cells under oxidative stress conditions. (B) Quantification of cell viability based on Calcein-AM/PI staining. (C) Representative phalloidin staining images showing cytoskeletal organization of HEI-OC1 cells. (D) CCK-8 assay evaluating the cytocompatibility of AD at different concentrations. Data are presented as mean $\pm$ SD (n = 3 independent experiments). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with the H₂O₂ group. Scale bars, 200 μ m and 100 μ m.

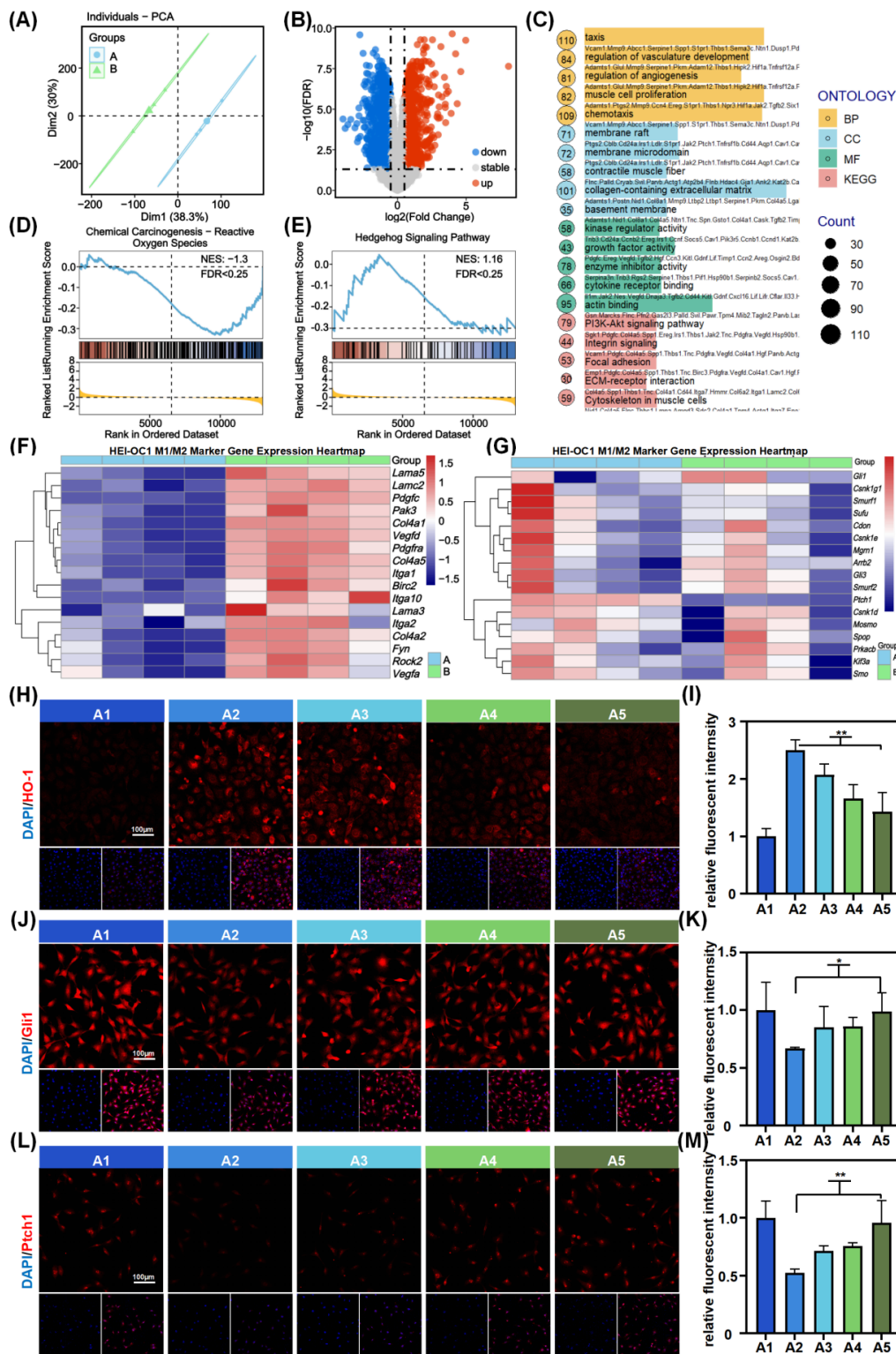


Figure 8

Transcriptomic and molecular analysis of AD-mediated cytoprotective remodeling in HEI-OC1 cells. (A) Principal component analysis (PCA) of transcriptomic profiles from different treatment groups. (B) Volcano plot showing differentially expressed genes. (C) GO and KEGG enrichment analyses of differentially expressed genes. (D) Gene set enrichment analysis (GSEA) of ROS-related pathways. (E) GSEA of Hedgehog signaling pathway. (F–G) Heatmaps of representative differentially expressed genes

associated with oxidative stress and Hedgehog signaling. (H–I) Representative immunofluorescence images and quantification of HO-1 expression. (J–M) Analysis of Gli1 and Ptch1 expression. Data are presented as mean $\pm$ SD ($n = 3$ independent experiments). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared with the H_2O_2 group. Scale bars, 100 μm .

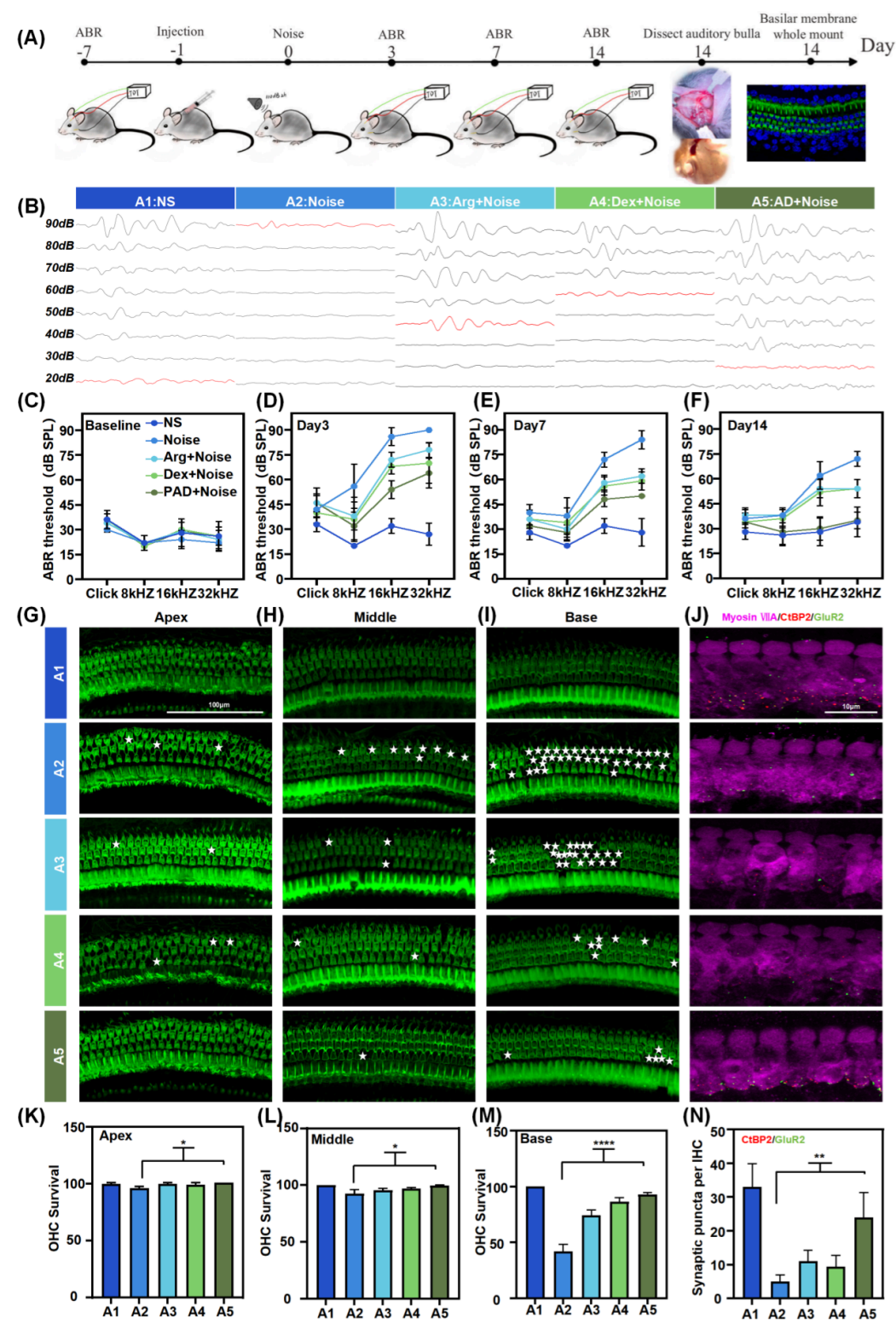


Figure 9

AD preserves auditory function, cochlear hair cells, and ribbon synapses in noise-induced hearing loss mice. (A) Experimental timeline for AD treatment and noise exposure in NIHL mice. (B–C) Baseline auditory brainstem response (ABR) thresholds before noise exposure. (D–F) ABR threshold shifts at 8, 16, and 32 kHz after different treatments. (G–I) Representative cochlear whole-mount images showing outer hair cell survival. (J) Representative immunofluorescence images of CtBP2/GluR2 staining for ribbon synapses. (K–M) Quantification of outer hair cell survival. (N) Quantification of paired ribbon synapses per inner hair cell. Data are presented as mean $\pm$ SD (n = 5 mice per group). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with the Noise group. Scale bars, 100 μ m and 10 μ m.

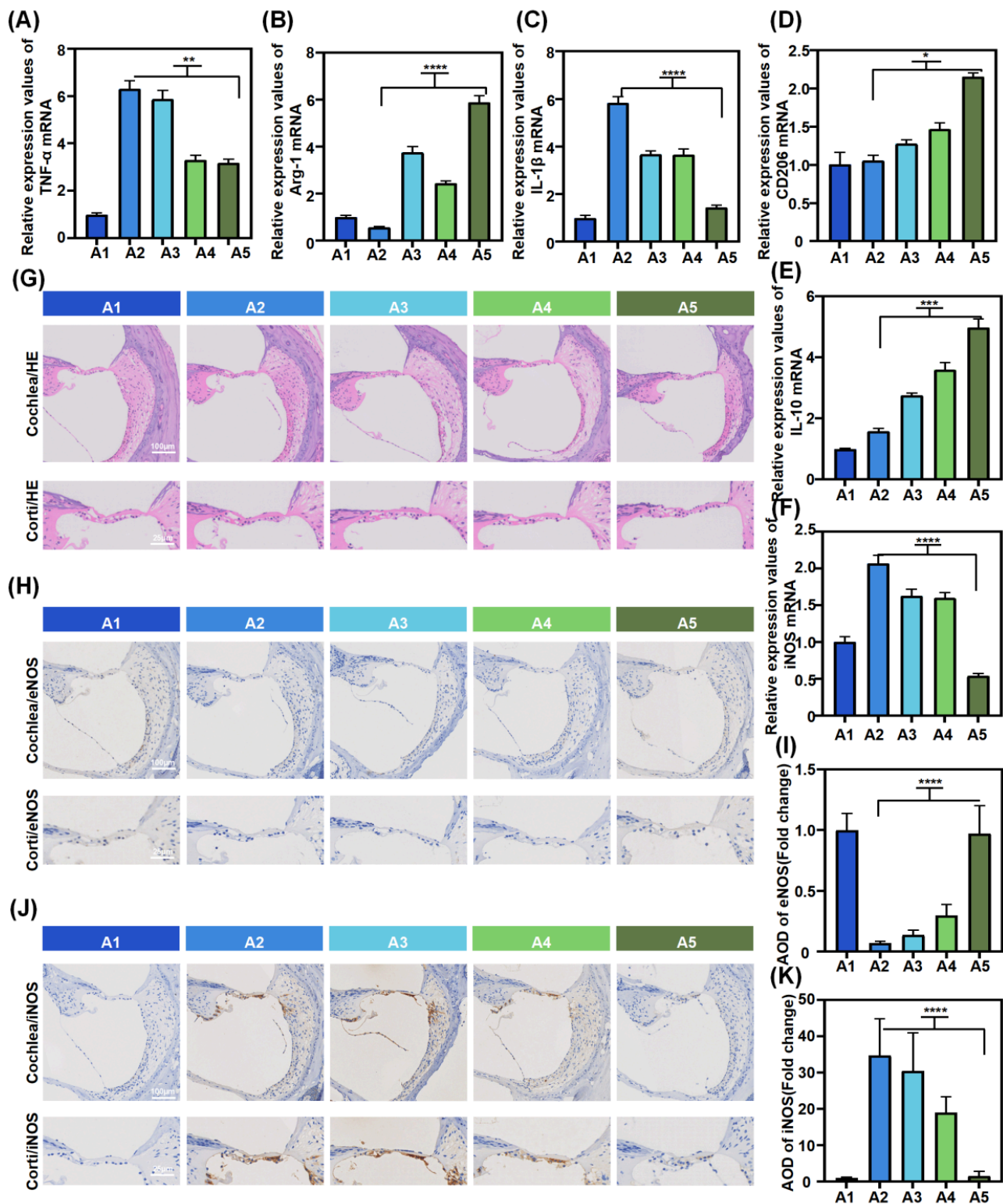


Figure 10

AD remodels the cochlear inflammatory-vascular microenvironment in vivo after noise exposure. (A–F) RT-qPCR analysis of TNF- α , IL-1 β , iNOS, Arg-1, CD206, and IL-10 expression in cochlear tissues. (G) Representative hematoxylin and eosin (H&E) staining images showing cochlear histopathological alterations. (H–I) Representative immunohistochemical staining images and quantitative analysis of eNOS expression. (J–K) Representative immunohistochemical staining images and quantitative analysis

of iNOS expression. Data are presented as mean $\pm$ SD (n = 5 mice per group). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with the Noise group. Scale bars, 100 μ m and 25 μ m.

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