Front. Med. 2022, 16(4): 637–650
https://doi.org/10.1007/s11684-021-0864-3

RESEARCH ARTICLE

Apigenin alleviates neomycin-induced oxidative damage via the Nrf2 signaling pathway in cochlear hair cells

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Abstract **Oxidative stress plays an important role in the pathogenesis of aminoglycoside-induced hearing loss and represents a promising target for treatment. We tested the potential effect of apigenin, a natural flavonoid with anticancer, anti-inflammatory, and antioxidant activities, on neomycin-induced ototoxicity in cochlear hair cells *in vitro*. Results showed that apigenin significantly ameliorated the loss of hair cells and the accumulation of reactive oxygen species upon neomycin injury. Further evidence suggested that the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway was activated by apigenin treatment. Disruption of the Nrf2 axis abolished the effects of apigenin on the alleviation of oxidative stress and subsequent apoptosis of hair cells. This study provided evidence of the protective effect of apigenin on cochlear hair cells and its underlying mechanism.**

Keywords apigenin; aminoglycosides; ototoxicity; oxidative stress; Nrf2 signaling pathway

Introduction

Drug-induced ototoxicity, such as due to aminoglycoside antibiotics and antitumor drugs like cisplatin and carboplatin, is one of the most common causes of hearing loss worldwide [1]. Different drugs can damage various cell types, including hair cells, supporting cells, and spiral ganglion cells [2]. Aminoglycosides mainly target hair cells once they penetrate into the endolymph through the blood–labyrinth barrier [3]. Previous studies [4–6] proposed that aminoglycosides might enter hair cells via a mechanoelectrical transducer channel or endocytosis. The exact mechanisms behind the ototoxicity of aminoglycosides remain unclear, but possible mechanisms include oxidative stress, autophagy, caspase-dependent apoptosis, and programmed cell necrosis that depends on receptor-interacting proteins [7]. Previous studies [8,9] suggested that oxidative stress might be the main contributor to aminoglycoside-induced hearing impairment. The production of reactive oxygen species (ROS) substantially increases upon neomycin or gentamicin injury in cochlear hair cells [10,11]. Various antioxidants, such as α-lipoic acid, coenzyme Q10, vitamin E, and salicylate, are reportedly effective in protecting hair cells against aminoglycoside ototoxicity [12,13]. Furthermore, autophagy activity considerably increases in auditory hair cells treated with neomycin. Abruption of the formation of autophagosome with the classic autophagy inhibitor 3-MA causes high ROS accumulation in hair cells, indicating the potent crucial role of autophagy in the antioxidative capacity of hair cells treated with aminoglycosides [14]. Moreover, the PINK1 signaling axis, which mediates mitochondrion autophagy, participates in ROS scavenging and thus in the regulation of ROS-induced apoptosis in auditory hair cells [15]. Therefore, targeting oxidative stress is a promising therapeutic strategy for aminoglycoside-induced ototoxicity.

Clinical trials and studies on the prevention and treatment of aminoglycoside-induced hearing loss (AIHL) have been conducted for years, but few effective medications for this condition have been developed [16]. Traditional medicines, such as mulberry extracts, are emerging alternatives for reducing ROS that can be potentially used as medications for drug-induced hearing loss [8]. Apigenin, a natural flavonoid that is present in a wide range of herbs and vegetables, is mainly extracted and purified from celery, parsley, and chamomile [17]. Apigenin reportedly has anticancer, anti-inflammatory, and

Received January 4, 2021; accepted April 25, 2021
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antioxidant activities [18]. Apigenin has been proved to be highly efficacious at inflammation suppression by inhibiting RelB, which is essential to the activation of the nuclear factor-κB transcription factor, at the transcriptional and translational levels; thus, apigenin can abolish the maturation of dendritic cells [19]. Apigenin can reportedly retard the development of chronic inflammatory diseases, such as atherosclerosis, by inhibiting the secretion of several pro-inflammatory cytokines in endothelial cells [20]. A recent study suggested that apigenin exerts favorable protective effects against pneumonia ischemia-reperfusion injury by reducing the expression levels of tumor necrosis factor-α, interleukine-6, and inducible nitric oxide synthase [21]. Importantly, apigenin can remarkably suppress the cellular content of ROS and subsequently lipid peroxidation by enhancing the expression of certain crucial antioxidative enzymes, such as catalase and glutathione peroxidase [14]. Moreover, it is revealed that certain genes, which relate to mitochondrial proliferation or are crucial for energy synthesizing, are upregulated with the administration of apigenin in neural and muscular degeneration disorders. Its antioxidant activity might also involve the downregulation of CD38 expression, a process that increases the intracellular NAD^+/NADH ratio and Sirt3-mediated mitochondrial antioxidative enzyme activity in a mouse model of diabetes-induced renal damage [22].

Owing to its anti-inflammatory and antioxidative effects, apigenin is a promising therapeutic compound for autoimmune and chronic inflammatory diseases, such as osteoarthritis and Alzheimer's disease [23]. However, the issue of whether apigenin can be utilized to prevent and treat AIHL remains unclear. Moreover, its possible mechanisms and its effective dose have yet to be determined. In this study, an aminoglycoside-ototoxicity model was established by isolating and culturing the cochlear epithelium *in vitro*. Apigenin was administered before and during neomycin treatment, which is a typical aminoglycoside antibiotic, to evaluate its possible protective effect against neomycin ototoxicity. Hopefully, our work would further reveal the pharmacological mechanism of apigenin and help shed light into the clinical benefit of this compound for patients with AIHL.

Materials and methods

Animals and ethics statement

C57/BL6 wild-type mice were bought from JSJ Experimental Animal Company Ltd. Atoh1-EGFP mice, the cochlear hair cells of which were labeled by EGFP fluorescence in the postnatal stage [24], were bred under specific and opportunistic pathogen-free conditions. All animals were treated in strict accordance with the Guidelines for Humane Treatment of Experimental Animals issued by the Ministry of Science and Technology of the People's Republic of China in 2006. All experiments were approved by Shanghai Medical Laboratory Animal Management Committee (License No. 2009-0082) and the Animal Ethics Committee of Fudan University.

Establishment of cochlear epithelium explants and drug treatment *in vitro*

The cochleae from postnatal mice were dissected and transferred into phosphate-buffered saline (Hyclone), and the extra surrounding brain tissues were carefully removed. After the removal of spiral ligament, the cochlear sensory epithelia were carefully adhered on transparent glass-slips coated with Cell-Tek (BD Bioscience) under a stereomicroscope. The cochlear epithelium explants were then cultured in DMEM/F12 (Hyclone) containing N2 (Life Technologies), B27 (Life Technologies), and 5 mg/mL ampicillin at 37 °C in an incubator with 5% CO_2 for 12 h before drug administration.

In vitro aminoglycoside-ototoxicity model was established by administering 1 mmol/L neomycin sulfate (Sigma-Aldrich, N6386) for 6 h. Neomycin was thoroughly removed from the culture medium, and then the explants were cultured for another 24 h before fixation. The possible protective effect of apigenin (Selleck, S2262) against neomycin damage was explored by administering it into the culture medium 2 h prior to neomycin treatment and continuously existed until the samples were harvested. The inhibitory effect of ML385 (Selleck, S8790) on nuclear factor erythroid 2-related factor 2 (Nrf2) signaling was verified by administering 5 μmol/L ML385 with apigenin 2 h prior to neomycin treatment.

Immunofluorescence

The samples were retrieved from the culture medium and fixed in 4% paraformaldehyde at room temperature. The tissues were then permeabilized and blocked with PBST (PBS with 1% dissolved Triton X-100) containing 10% donkey serum for 1 h. Well-prepared samples were immersed in PBST with 1% donkey serum and certain primary antibodies overnight at 4 °C. The primary antibodies used in this study included anti-myosin VIIA (Myo7a) antibody (Proteus Biosciences; 25-6790; 1:1000 dilution), anti-parvalbumin antibody (Sigma-Aldrich; P3088; 1:1000 dilution), and anti-Nrf2 antibody (Abcam; ab137550; 1:200 dilution). The samples were rinsed three times with PBS and then incubated with the corresponding secondary antibodies conjugated with various fluorescence, protected from light, for 12 h at 4 °C. The samples were labeled with DAPI (Sigma-Aldrich, D9542) for nuclei staining and then detected and recorded using a confocal fluorescence microscope (Leica Microsystems, SP8).

Apoptosis detection with TUNEL staining

The apoptosis of hair cells induced by neomycin administration was detected with a TUNEL Kit (Roche, 11772457001) following the manufacturer's instructions. DAPI and anti-Myo7a immunostaining were also performed to locate the nuclei of the hair cells. The samples were prepared and detected in the same manner as described above.

ROS staining with MitoSOX and DCFH-DA

The presence of ROS in the hair cells after neomycin administration was detected with a MitoSOX Red mitochondrial superoxide indicator (Invitrogen, M36008) in accordance with the manufacturer's instructions. DAPI and anti-Myo7a immunostaining were also performed to locate hair cells. The samples were prepared and detected in the same manner as described above.

DCFH-DA staining was used to detect intracellular ROS levels. The samples were incubated with DCFH-DA (Beyotime, S0033S) for 30 min after neomycin treatment and then washed with culture medium twice for 5 min each. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich, 94403) before observation.

Gentamicin conjugated by Texas Red uptake experiments

Gentamicin conjugated by Texas Red (GTTR) was diluted to the final working concentrations of 50 μmol/L as previously described [25]. Cochlear explants from the Atho1-EGFP mice were preincubated with 20 μmol/L apigenin for 2 h and then co-incubated with the GTTR for 30 min. The cochlear explants incubated in the media containing Texas Red alone were used as controls.

Study on neomycin efficacy

The interfering effect of apigenin on the antibiotic efficacy of neomycin was assessed as previously reported [26]. First, 5 mm filter discs were soaked overnight in PBS, 20 μmol/L apigenin, 1 mmol/L neomycin, 20 μmol/L apigenin + 1 mmol/L neomycin, or 50 μmol/L apigenin + 1 mmol/L neomycin. Subsequently, *Escherichia coli* HST08 strain was plated in agar plates, and the filter discs were leaned on top of them. A filter disc from each group was placed on the same agar plate to minimize error. The plates were incubated overnight and photographed, and the inhibitory area was calculated for each condition and expressed as square millimeters.

qPCR

Total RNA was extracted from different groups of cochlear explants treated with neomycin for 3 or 6 h, with or without apigenin or ML385, by using Trizol reagent (Invitrogen, 15596018). cDNA was synthesized using the 1st Strand cDNA Synthesis Kit (Takara, 6210A). qPCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems) by using the TB Green PrimeScript qPCR Kit (Takara, RR820A). The sequences of all primers are listed in Table 1. Actb was used as a housekeeping gene for endogenous reference. Relative gene expression compared with that of Actb was quantified via the $2^{-\Delta\Delta}$CT method.

Cell counting and statistical analysis

Immunostaining-positive cells were quantified by selecting nine separate segments from the apical to the basal turn along the entire cochlea (approximately 4.0, 5.6, and 8.0 kHz in the apical turn; 11.3, 16.0, and 22.6 kHz in the middle turn; and 32.0, 45.2, and 64.0 kHz in the basal turn). Data were presented as the mean ± SD. Student's *t*-test and ANOVA analysis followed by Bonferroni test were used for two-group or multiple-group comparison, respectively. P value < 0.05 was considered significant ($*$ represents P value < 0.05, $**$ represents P value < 0.01, $***$ represents P value < 0.001, $****$ represents P value < 0.0001).

Results

Apigenin protected cochlear hair cells from neomycin ototoxicity

Apigenin, a natural compound derived from 2-phenylchromone (Fig. 1A), has been verified to have cellular

Table 1 Primers used in qPCR

Primer	Sequence (5′–3′)
Actb forward	GGCTGTATTCCCCTCCATCG
Actb reverse	CCAGTTGGTAACAATGCCATGT
Nrf2 forward	AGATGACCATGAGTCGCTTGC
Nrf2 reverse	GCCAAACTTGCTCCATGTCC
HO-1 forward	GCTAGCCTGGTGCAAGATACT
HO-1 reverse	TGGGGGCCAGTATTGCATTT
Gclc forward	CCTCCAGTTCCTGCACATCT
Gclc reverse	GTCTCAAGAACATCGCCTCC
Gclm forward	GGAATGCACCATGTCCCATG
Gclm reverse	AGCCATGATCACAGAGTCCA
Gpx1 forward	GGACTACACCGAGATGAACG
Gpx1 reverse	TCTCACCATTCACTTCGCAC
Sod1 forward	GGGTTCCACGTCCATCAGTA
Sod1 reverse	GGTCTCCAACATGCCTCTCT
Sod2 forward	TGTTACAACTCAGGTCGCTCT
Sod2 reverse	CTCCCACAGACACGGCTG

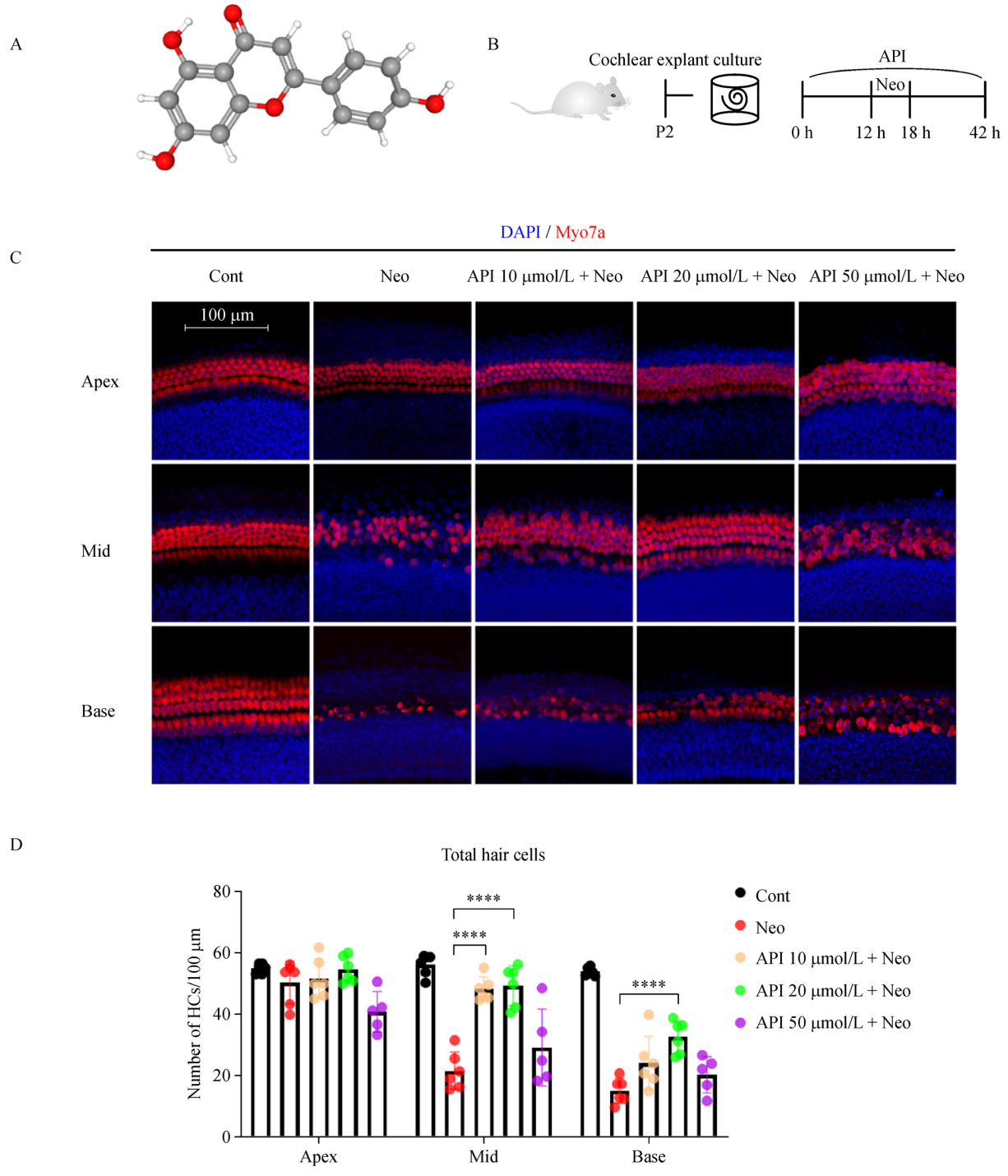

Fig. 1 Apigenin protected cochlear hair cells from neomycin ototoxicity. (A) Chemical structure of apigenin. (B) Diagram of the experimental process. Cochlear sensory epithelia were isolated from C57/BL6 neonatal mice and cultured *in vitro* for 12 h followed by neomycin treatment at the concentration of 1 mmol/L for 6 h. The cochlear explants recovered for another 24 h before they were harvested with the withdrawal of neomycin. (C) Representative images of immunofluorescence with anti-Myo7a antibody (red) and DAPI (blue) from the apical, middle, and basal turns of the cochleae. Myo7a was used as a marker of cochlear hair cells. The samples were divided into five groups: Cont (control), Neo (1 mmol/L neomycin only), and neomycin co-treated with different concentrations of apigenin. Scale bar = 100 μm. (D) Quantification of Myo7a-positive hair cells per 100 μm from different groups. Data are shown as mean ± SD. **** $P < 0.0001$. $n = 6$.

protective activity against many diseases. However, its activity against aminoglycoside ototoxicity lacks evidence. Thus, the tissue model was first established by culturing the cochlear epithelium *in vitro* from newborn wild-type C57 mice and by adding neomycin into the medium (Fig. 1B). Previous studies [14,27,28] confirmed that neomycin treatment can result in acute disruption of the physiologic functions of hair cells and subsequent dispatch from the supporting cells or cell lysis. Interestingly, the hair cells from the middle and the basal turns of the cochleae were more susceptible to neomycin ototoxicity than those from the apical turns. Different concentrations of apigenin were administered to explore its possible effect on aminoglycoside-induced ototoxicity. The hair cells were stained with anti-Myo7a antibody, and their amount was calculated (Fig. 1C and 1D). Results showed that 10 and 20 μmol/L apigenin exerted a protective effect against neomycin-induced cochlear hair cell death as demonstrated by the substantially higher numbers of hair cells from the middle and basal turns, especially in the 20 μmol/L apigenin co-treated group. However, 50 μmol/L apigenin did not have a notable protective effect for the hair cells. Therefore, 20 μmol/L was considered the best therapeutic concentration for apigenin.

The protective effect of apigenin on the hair cells observed herein might be attributed to the interaction between apigenin and neomycin that affected the activity of the antibiotic, thereby reducing the extent of damage to the hair cells. This phenomenon was explored by performing a disc diffusion test in which the *E. coli* HST08 strain was exposed to neomycin (1 mmol/L) alone or in the presence of apigenin (20 μmol/L or 50 μmol/L), and the inhibitory area was calculated after incubating overnight (Fig. 2A). Results showed that apigenin did not affect the antibacterial activity of neomycin (Fig. 2B).

Additionally, GTTR was used to assess the entry of the aminoglycosides into the cochlear hair cells in the presence of apigenin. Results showed that incubation with 20 μmol/L apigenin had no significant effect on GTTR uptake (Fig. 2C and 2D), suggesting that the protective effect of apigenin was not the result of the inhibition of aminoglycoside uptake but rather than the interference with intrinsic death pathway in the hair cells.

Apigenin suppressed the oxidative stress and apoptosis induced by neomycin in the hair cells

ROS accumulation, which is the primary indicator of oxidative status within cells, contributes to the pathological process of neomycin-induced hair cell death. The question of whether apigenin can reduce ROS generation or accumulation in injured hair cells was explored herein. MitoSOX Red is a cytomembrane permeable dye that can selectively reside in the mitochondria of living cells. Once oxidized by ROS, red fluorescence is activated and maintained even after fixation. MitoSOX Red immunofluorescence staining was immediately performed after 6 h of injury (Fig. 3A). The numbers of MitoSOX and Myo7a double labeled cells were considerably higher in the middle and basal turns of the cochlear epithelia co-treated by 10 μmol/L or 20 μmol/L apigenin and neomycin compared with those in the neomycin-only group (Fig. 3B and 3C). This result suggested that apigenin effectively suppressed neomycin-induced oxidative stress. However, 50 μmol/L apigenin did no exert a notable inhibitory effect on neomycin-induced increase in mitochondrial ROS (Fig. 3B and 3C). DCFH-DA was also used to detect ROS levels in the hair cells upon neomycin challenge. Intracellular ROS can oxidize nonfluorescent DCFH to generate fluorescent DCF and thus display the level of intracellular ROS. In this study, three typical regions (24, 32, and 48 kHz) in the cochlear epithelia were selected for fluorescence quantitative analysis (Fig. 3D). Consistent with the MitoSOX staining results, DCFH-DA staining results showed that 20 μmol/L apigenin considerably inhibited the increase in intracellular ROS level induced by neomycin treatment (Fig. 3E), further confirming that apigenin effectively suppressed neomycin-induced oxidative stress. Excessive ROS accumulation can induce the opening of mitochondrial permeability transition pore, resulting in the release of calcium, cytochrome c (cyto-c), and apoptosis-inducing factors. The number of apoptotic cells confirmed by TUNEL staining was then counted. TUNEL staining can detect the 3-OH of cleaved DNA fragments, which is a hallmark of apoptosis. Apigenin substantially attenuated neomycin-induced apoptosis in the hair cells, as proved by the quantification of Myo7a and TUNEL double-positive hair cells (Fig. 4A–4C).

Apigenin upregulated Nrf2 signaling in the cochlear explants

The present study confirmed the protective effect of apigenin against neomycin-induced hair cell injury. Accordingly, the underlying potent mechanism behind this effect was investigated. Xu *et al*. [29] demonstrated that in the cell model of age-related macular degeneration, apigenin attenuates the pathological deterioration targeting the Nrf2 axis and the subsequent downstream antioxidant genes. The potential activating effect of apigenin on the Nrf2 pathway was also suggested by another study on mouse retina [30]. Given that Nrf2 is widely acknowledged as a key regulator of antioxidative pathways in mammal cells [31], we hypothesized that the protective activity of apigenin in inner ear is also relevant to the Nrf2 pathway because apigenin administration remarkably reduced the oxidative stress detected in the hair cells. qPCR was performed to evaluate the expression level of Nrf2 and its downstream target genes, including heme oxygenase-1 (HO-1), superoxide dismutase (Sod),

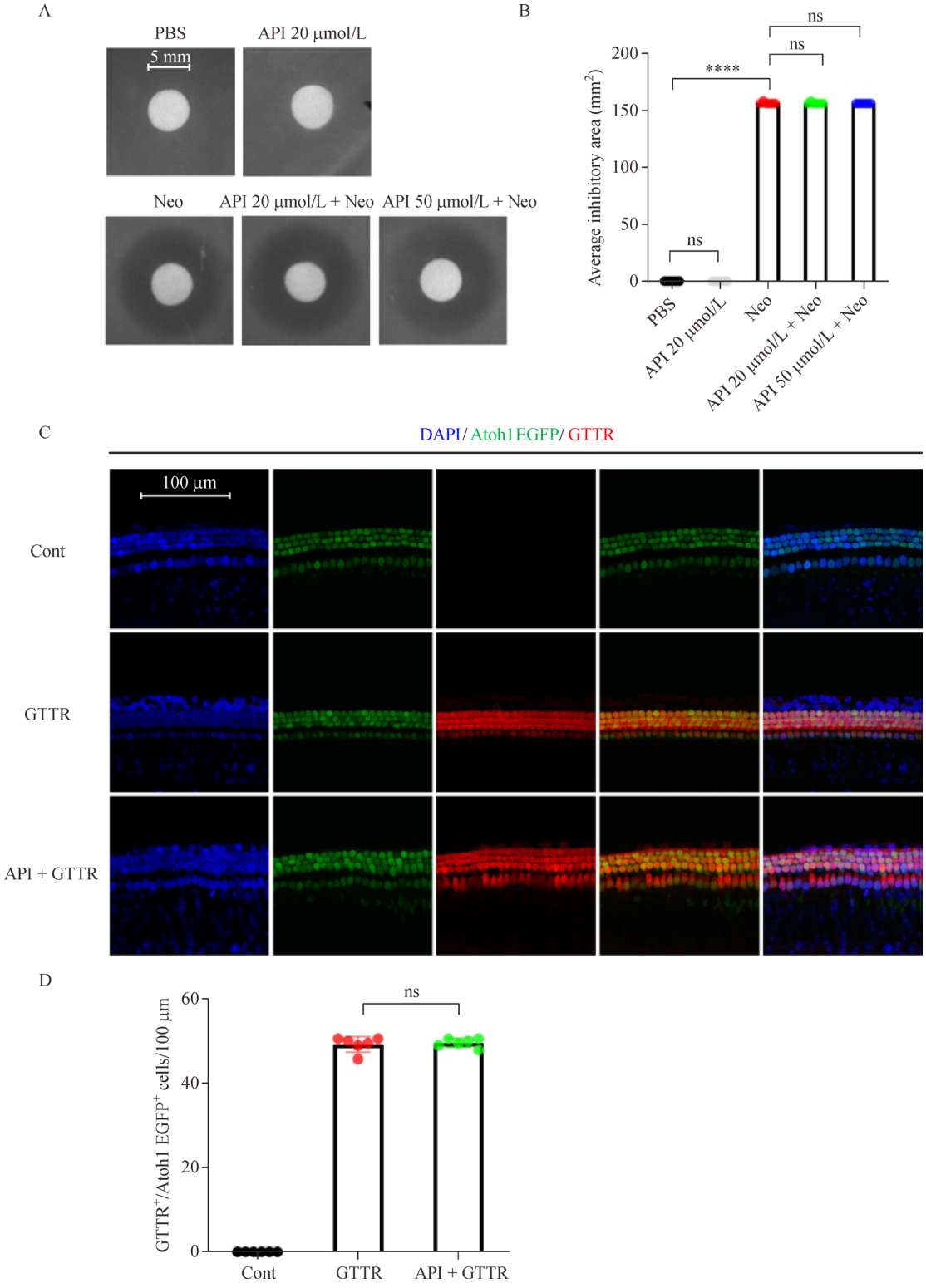

Fig. 2 Apigenin did not affect the antibiotic efficacy of neomycin and the uptake of aminoglycosides. (A) Representative images of the disc diffusion test for groups of PBS, 20 μmol/L API, 1 mmol/L neomycin, 20 μmol/L API + 1 mmol/L neomycin, and 50 μmol/L API + 1 mmol/L neomycin. Scale bar = 5 mm. (B) Statistical data of inhibitory areas. (C) Representative images of Atoh1-EGFP (green), Texas Red-conjugated gentamycin (GTTR, red), and DAPI (blue) fluorescence in the mid-base turns of the cochleae. GTTR was used to assess the uptake of aminoglycosides. Atoh1-EGFP-positive cells were cochlear hair cells. The samples were divided into three groups: Cont (control), GTTR (50 μmol/L GTTR only), and API + GTTR (20 μmol/L apigenin + 50 μmol/L GTTR). (D) Statistical data of Atoh1-EGFP- and GTTR double-positive cells in the cochlear middle and basal turns. Scale bar = 100 μm. Data are shown as mean ± SD. ns, no significant difference. $n = 6$.

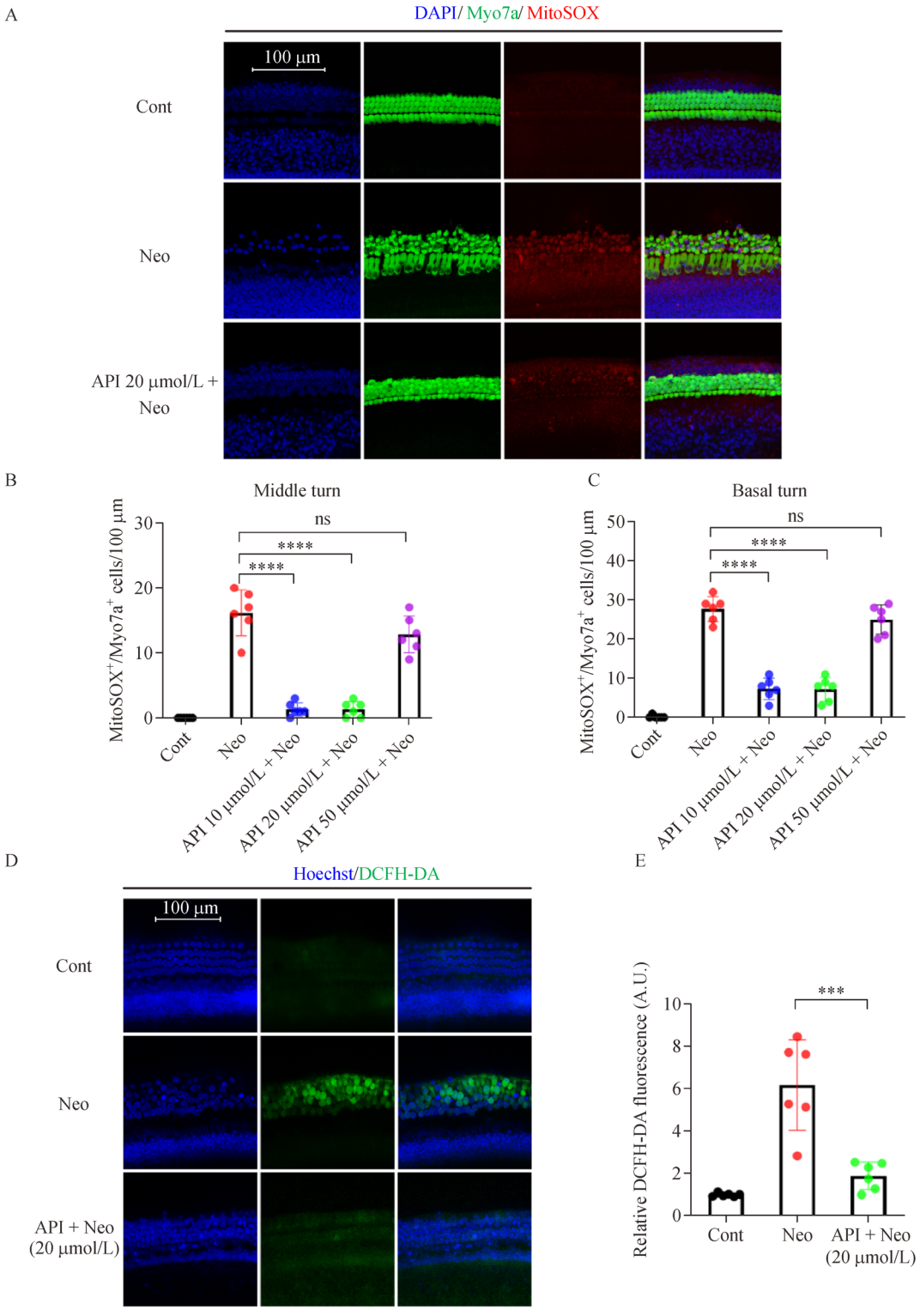

Fig. 3 Apigenin suppressed oxidative stress induced by neomycin in the hair cells. (A) Representative images of staining with anti-Myo7a antibody (green), MitoSOX (red), and DAPI (blue) from the middle turns of the cochleae. MitoSOX Red was used as a marker in evaluating ROS level. Scale bar = 100 μm. (B and C) Quantification of Myo7a and MitoSOX double-positive hair cells per 100 μm from the middle and basal turns. The samples were divided into five groups: Cont (control), Neo (1 mmol/L neomycin only), API 10 μmol/L + Neo (10 μmol/L apigenin + 1 mmol/L neomycin), API 20 μmol/L + Neo (20 μmol/L apigenin + 1 mmol/L neomycin), and API 50 μmol/L + Neo (50 μmol/L apigenin + 1 mmol/L neomycin). Data are shown as mean ± SD. **** $P < 0.0001$. $n = 6$. (D) Representative images of DCFH-DA (green) and Hoechst (blue) staining from the mid-base turn of the cochleae. DCFH-DA was used as an indicator of intracellular ROS. The samples were divided into three groups: Cont (control), Neo (1 mmol/L neomycin only), and API + Neo (20 μmol/L apigenin + 1 mmol/L neomycin). Scale bar = 100 μm. (E) Fluorescent quantitation of DCFH-DA from the mid-base turn. Data are shown as mean ± SD. *** $P < 0.001$. $n = 6$.

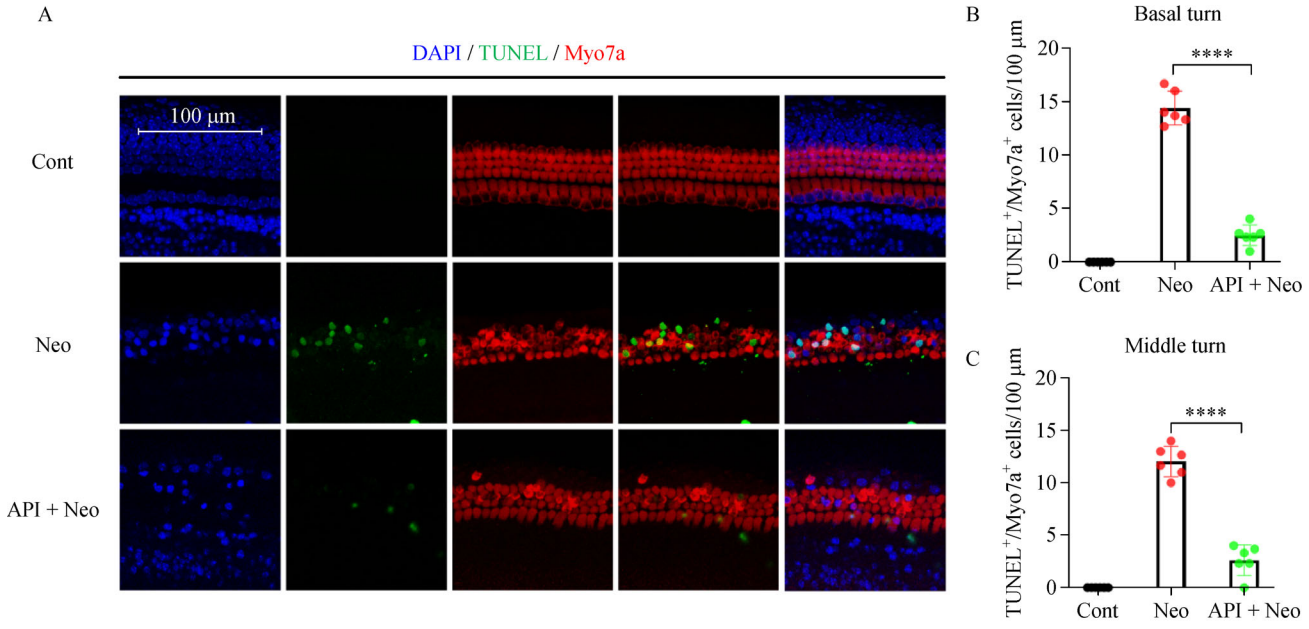

Fig. 4 Apigenin attenuated neomycin-induced apoptosis in the hair cells. (A) Representative images of staining with anti-Myo7a antibody (red), TUNEL (green), and DAPI (blue) from the middle turn of the cochleae. TUNEL staining confirmed the presence of apoptotic cells. The samples were divided into three groups: Cont (control), Neo (1 mmol/L neomycin only), and API + Neo (20 μmol/L apigenin + 1mmol/L neomycin). Scale bar = 100 μm. (B and C) Quantification of Myo7a and TUNEL double-positive hair cells per 100 μm. Data are shown as mean ± SD. **** $P < 0.0001$. $n = 6$.

glutathione peroxidase (Gpx), glutamate–cysteine ligase modifier (Gclm), and glutamate–cysteine ligase catalytic (Gclc). As shown in Fig. 5A and 5B, apigenin apparently elevated the mRNA abundance of Nrf2 and upregulated the expression of the antioxidative enzymes, including Gclc, Gclm, and Sod2, in the neomycin-treated hair cells. Our hypothesis was also confirmed by the results of immunostaining with anti-Nrf2 antibody (Fig. 5C). However, the expression of Nrf2 was not apparently enhanced upon oxidative stimulation as no statistically significant difference was observed between the neomycin-treated group and the control group. These data suggested that Nrf2 upregulation occurred in the hair cells upon apigenin treatment, leading to the transcription of antioxidative genes.

Nrf2 signaling mediated the protective effect of apigenin against neomycin-induced oxidative stress and apoptosis

The issue of whether the antioxidative activity of apigenin against neomycin ototoxicity depends on Nrf2 was explored using the NRF2 inhibitor ML385. This inhibitor not only reduced the protein content of NRF2 by suppressing the translational activity of Nrf2 but also directly inhibited the expression of the downstream target genes by blocking the combination of Nrf2 with its promoters. The expression levels of Nrf2 and its target genes, such as Gclc, Gclm, and Sod2, were substantially reduced by the addition of ML385 at the transcriptional and translational levels (Fig. 5B and 5C). Furthermore, the effects of apigenin on oxidative stress amelioration and apoptosis inhibition were considerably abrogated by ML385 in the hair cells challenged with neomycin (Fig. 6A–6F). Notably, the inhibition efficacy of ML385 was not as high as expected, as illustrated by the results of qPCR (Fig. 5B), despite the fact that ML385 sufficiently disrupted ROS accumulation and apoptosis. Quantification of the surviving hair cells showed that the difference in the cochlear middle and basal turns between the API + Neo and ML385 + API + Neo groups was statistically significant (Fig. 7A and 7B). Collectively, these results suggested that apigenin relieved aminoglycoside-induced ototoxicity by reducing oxidative stress and inhibiting apoptosis via the Nrf2 axis (Fig. 8).

Discussion

Thus far, few effective medications have been approved for the treatment of AIHL [32]. Several pathways, including oxidative stress and chronic inflammation, seem to play important roles in the pathogenesis of AIHL, and they represent possible targets for AIHL treatment [33]. In the present study, the hair cells exposed to neomycin suffered from severe injury with excessive ROS accumulation and

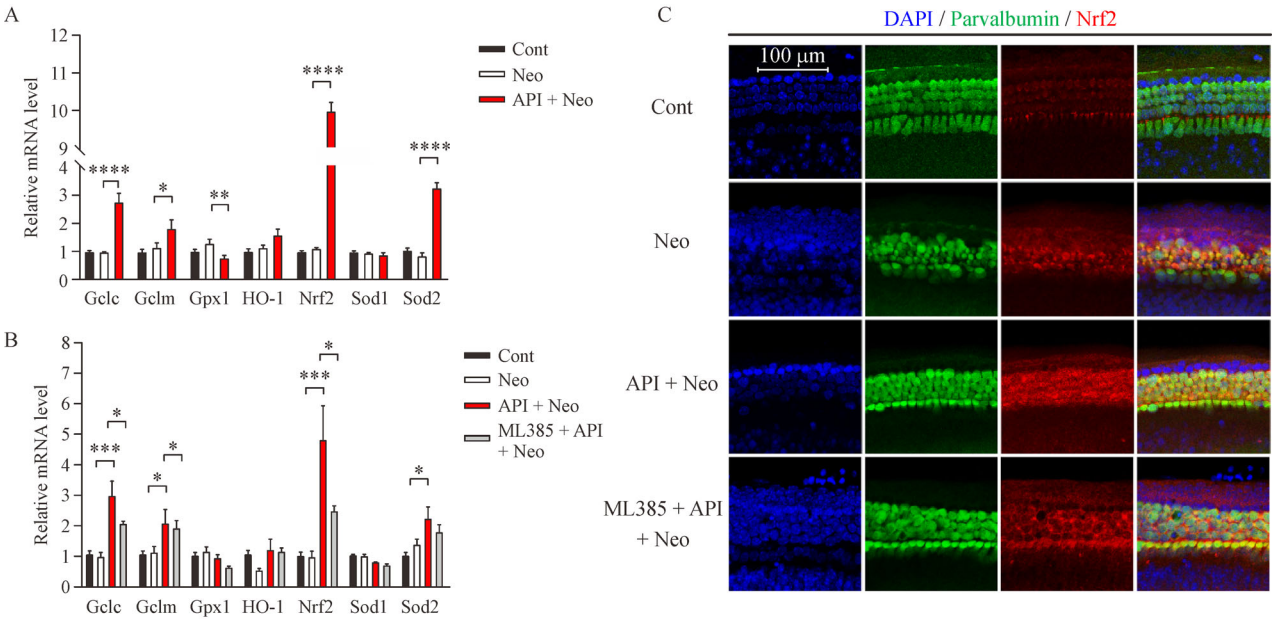

Fig. 5 Apigenin upregulated Nrf2 signaling in the cochlear explants. (A and B) Relative mRNA levels of antioxidant-related genes in the cochlear explants after 3 (A) or 6 (B) h of neomycin injury with or without apigenin. qPCR data were normalized to Actb and presented as the fold of control levels. The samples were divided into four groups: Cont (control), Neo (1 mmol/L neomycin only), API + Neo (20 μmol/L apigenin + 1 mmol/L neomycin), and ML385 + API + Neo (5 μmol/L ML385 + 20 μmol/L apigenin + 1 mmol/L neomycin). Data were shown as mean ± SD. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. (C) Representative images of immunostaining with anti-parvalbumin antibody (green), anti-Nrf2 antibody (red), and DAPI (blue). Parvalbumin was used as a marker of cochlear hair cells. Scale bar = 100 μm.

activation of apoptotic cell death. ROS, the principal cellular oxidant produced mostly by the electron transport in the mitochondria [34], is essential to multiple physiologic functions, such as phagocytosis of neutrophils as a defensive molecule [35], as well as to cell growth and stress responses as a second messenger [36,37]. Nevertheless, a subtle balance must be maintained for redox homeostasis via the meticulous regulation of oxidative and antioxidative substances [38]. By contrast, excessive ROS accumulation can damage the basic structure of proteins, lipids, DNA, and other biomacromolecules [39,40]. The mitochondrion is the main target of ROS overload. The bilayer membrane of the mitochondria has two oxidative-sensitive sites, both of which are prone to ROS oxidation: one is pyridine nucleotide binding site, which has high affinity to NAD(H) and NADP(H); and the other is glutathione binding site. The cyto-c released from the mitochondria into the cytoplasm is involved in the formation of apoptosomes, and it combines with apoptosis-related factor-1 and caspase-9 precursor, thereby further activating caspase-3/7 to induce apoptosis [41]. Furthermore, excessive ROS accumulation can decouple the mitochondrial electron transport chain; rupture normal mitochondrial functions, such as oxidative phosphorylation and ATP generation; promote the expression of the pro-apoptotic protein Bax (B cell lymphoma 2 associated X); and finally contribute to apoptosis [42].

ROS balance is regulated by their production and scavenging; the latter is mediated by antioxidant enzymes, including catalase and SOD, which detoxify ROS and reduce ROS-dependent cell death [43,44]. Consistent with the reports of previous studies [29,30,45], apigenin increased not only the mRNA level of Nrf2 but also the protein content of Nrf2, leading to the transcription of several antioxidant genes in the hair cells treated with neomycin (Fig. 5). Nrf2 is an important redox-sensitive transcription factor that is expressed in the cells of various tissues, such as liver, kidneys, and inner ear [46,47]. Under physiologic conditions, Nrf2 is anchored in the cytoplasm by Keap1. As the substrate of Cul3-dependent E3 ubiquitin ligase complex, Keap1 can promote the ubiquitination of Nrf2 and subsequent degradation by proteasomes. However, under oxidative stress, Nrf2 dissociates from Keap1 and quickly translocates into the nucleus to activate the expression of antioxidant enzymes, such as HO-1, catalase, SOD, and GPx [48,49]. For example, HO-1 has been comprehensively studied about its antioxidative role in catalyzing the decomposition of heme into ferrous, carbon monoxide, and biliverdin, and the biliverdin can effectively scavenge peroxides, peroxynitrite, and superoxide

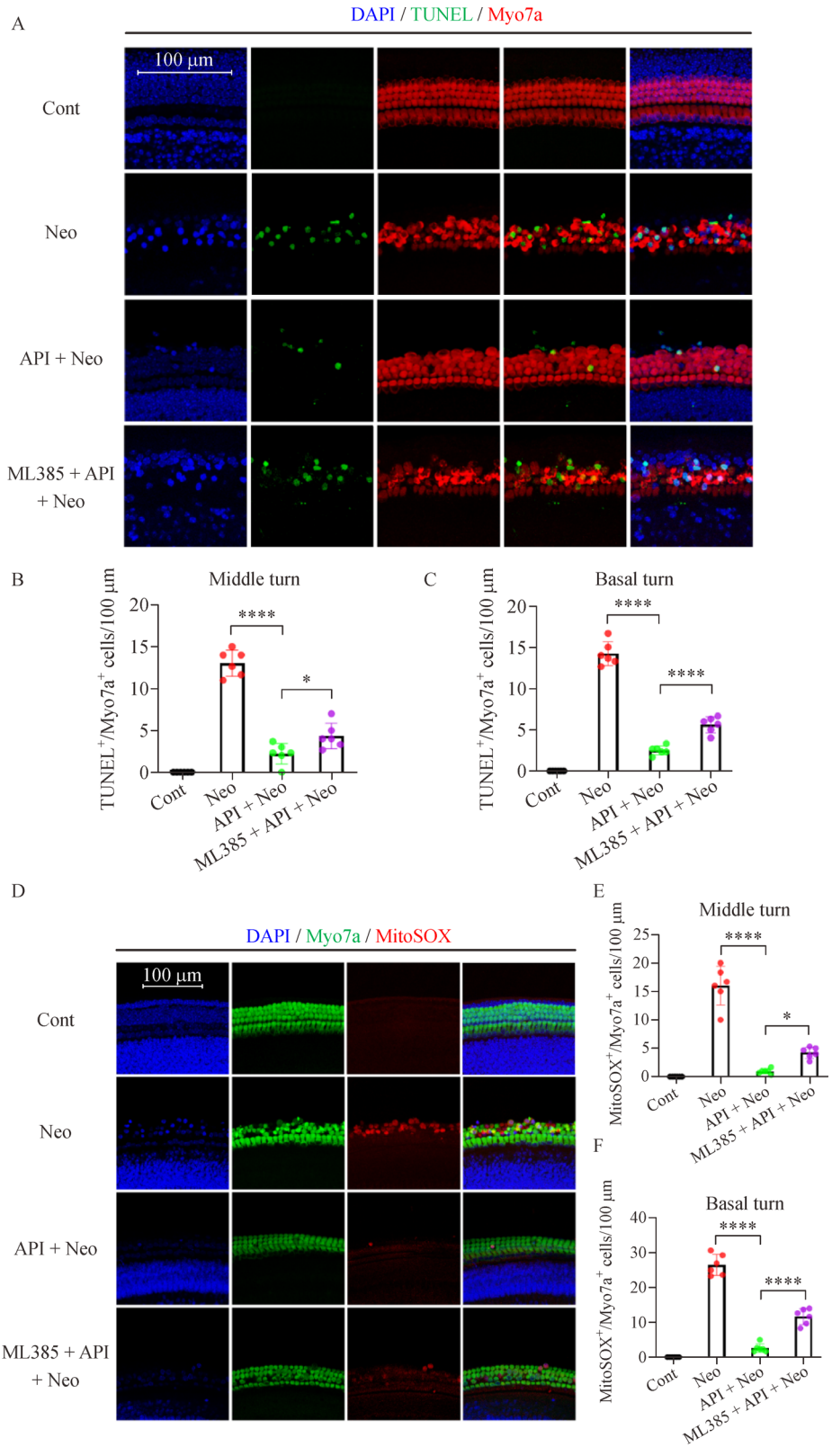

Fig. 6 Nrf2 signaling mediated the protective effect of apigenin against neomycin-induced oxidative stress and apoptosis. (A) Representative images of immunostaining with anti-Myo7a antibody (red), TUNEL (green), and DAPI (blue) from the middle turns of the cochleae. (B and C) Quantification of Myo7a and TUNEL double-positive hair cells from the middle and basal turns of the cochleae. (D) Representative images of immunostaining with anti-Myo7a antibody (green), MitoSOX (red), and DAPI (blue) from the middle turns of the cochleae. (E and F) Quantification of Myo7a and MitoSOX double-positive hair cells per 100 μm from the middle and basal turns of the cochleae. Scale bar = 100 μm. Data are shown as mean ± SD. *$P < 0.05$, **** $P < 0.0001$. $n = 6$.

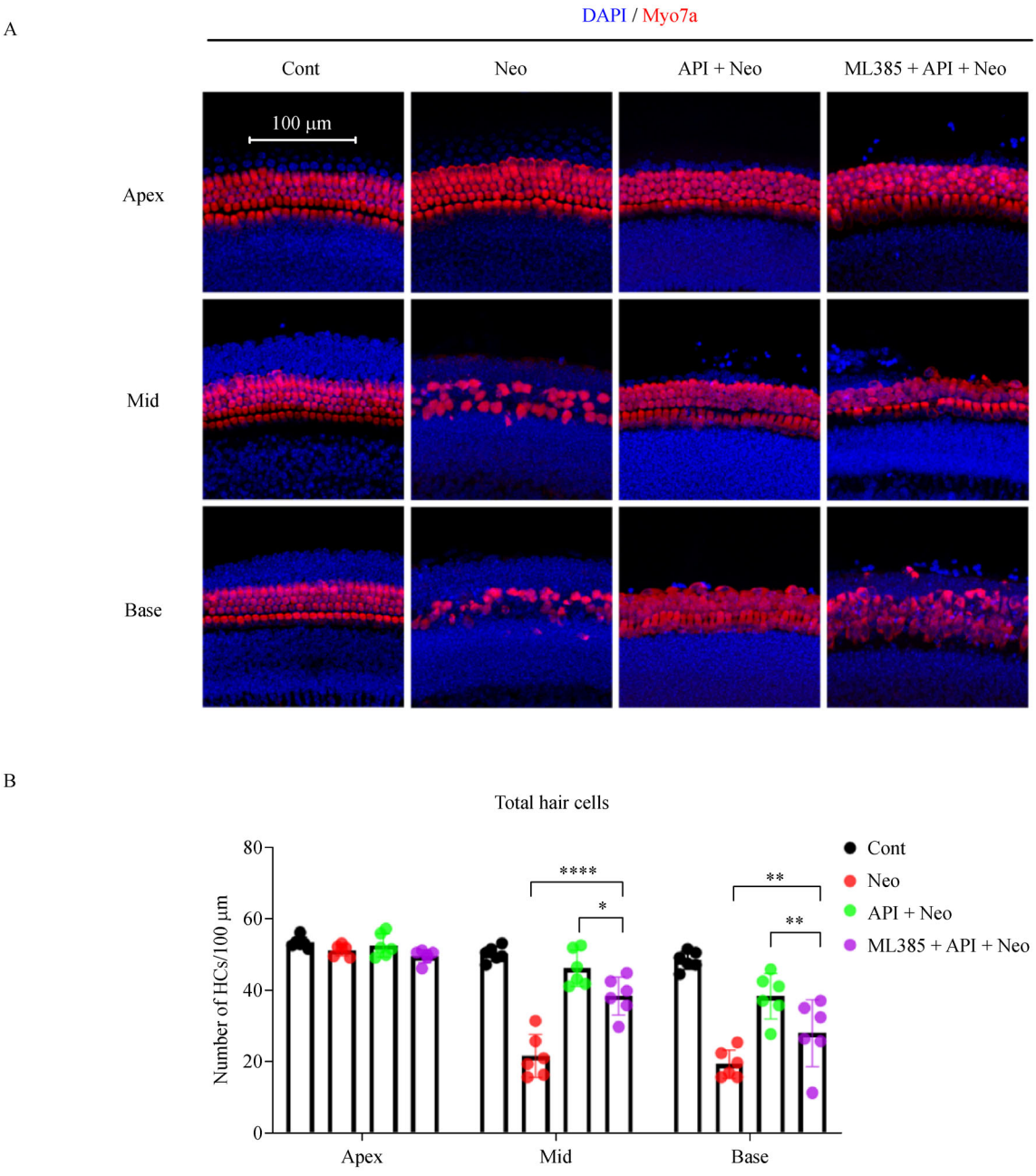

Fig. 7 Apigenin alleviated the neomycin-induced loss of hair cells by targeting Nrf2 signaling. (A) Representative images of immunofluorescence with anti-Myo7a antibody (red) and DAPI (blue) from the apical, middle, and basal turns of the cochleae. Scale bar = 100 μm. (B) Quantification of Myo7a-positive hair cells in the apical, middle, and basal turns of the cochleae from different groups. Data are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. $n = 6$.

radicals [50]. Nrf2-knockout mice showed greater susceptibility to gentamicin-induced ototoxicity and progressive hearing loss at early ages than control mice [51], with spontaneous loss of auditory spiral ganglion, indicating the loss of resistance to environmental toxicity and age-related ROS overload. Moreover, activation of Nrf2 signaling protects hair cells not only from aminoglycoside injury but also from other ototoxic drugs, such as cisplatin injury, by reducing ROS [52].

Although oxidative stress is the primary component of the mechanism behind aminoglycoside-induced hair cell injury, autophagy [14] and inflammation [53] are also involved in the pathophysiology of AIHL. Interestingly, aside from enhancing cellular defense ability against oxidative stress, the therapeutic potential of apigenin has also been proved to be related to inflammation and

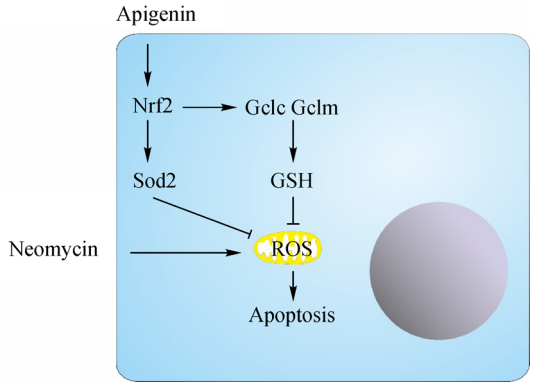

Fig. 8 Schematic of the underlying mechanism of the protective effect of apigenin against neomycin ototoxicity. Apigenin relieved aminoglycoside-induced ototoxicity by reducing oxidative stress and inhibiting apoptosis by activating the Nrf2 axis.

autophagy. Apigenin can reportedly suppress inflammatory stress by activating anti-inflammatory signaling pathways, such as the p38/MAPK and PI3K/Akt signaling pathways, as well as by blocking the expression of pro-inflammation cytokines [54,55]. Apigenin can also modulate autophagy-related proteins, such as LAMP-1, ATG5, and p62, to protect cardiomyocytes from inflammatory and oxidative injury [56]. The issue of whether the protective effect of apigenin against aminoglycoside ototoxicity is related to its anti-inflammation and pro-autophagy function requires further exploration.

The protective effect of apigenin is currently being investigated in multiple clinical trials. A formulation containing apigenin applied twice a day for 1 year was found to improve the cognitive performance of patients suffering from Alzheimer's disease [57]. Supplementation of apigenin-rich chamomile oil for 3 weeks was observed to reduce the occurrence and extent of pain in patients with knee osteoarthritis [58]. The present study helps in expanding the clinical indications for apigenin by providing an in-depth understanding of its pharmaceutical mechanism. Apigenin also reportedly substantially inhibits cell proliferation and promotes apoptosis in esophagus cancer tissues in a dose-dependent manner, suggesting that the mechanisms behind its dynamic pro-survival or pro-apoptosis roles are complicated and poorly understood [59,60].

Apigenin is a potential preventative and therapeutic medicine for hearing disorders. However, the appropriate clinical dose still requires further study. Several concerns on the clinical application of apigenin must be addressed. First, the poor water solubility of apigenin might reduce its oral bioavailability [61]. Second, its ability to penetrate the blood–labyrinth barrier is also a factor that affects its efficacy. Nevertheless, apigenin has been resynthesized via multiple target-directed ligand strategies to acquire pharmaceutical property with a better water-solubility and a higher blood–brain barrier permeability while retaining its antioxidant activity [62]. These concerns might also be resolved through nanotechnology [63] or other manipulations, such as liposomal preparations.

In conclusion, this study proved that apigenin relieves aminoglycoside-induced ototoxicity by reducing oxidative stress and inhibiting apoptosis via the Nrf2 axis. These findings might provide new insights into the treatment of hearing impairment induced by ototoxic drugs.

Acknowledgements

This work was supported by the National Key R&D Program of China (No. 2017YFA0103900), the National Natural Science Foundation of China (Nos. 81771010 and 81570911), and the Nature Science Foundation (No. 17ZR1404600) from Shanghai Science and Technology Committee.

Compliance with ethics guidelines

Gaogan Jia, Huanyu Mao, Yanping Zhang, Yusu Ni, and Yan Chen declare that there is no conflict of interest regarding the publication of this article. This study was approved by the Institutional Research Ethics Committee of the Eye & ENT Hospital of Fudan University.

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