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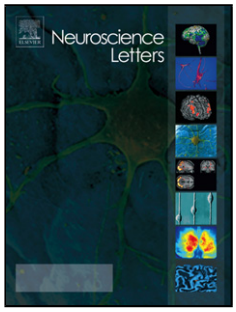

Apelin protects auditory cells from cisplatin-induced toxicity *in vitro* by inhibiting ROS and apoptosis

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Apelin protects auditory cells from cisplatin-induced toxicity *in vitro* by inhibiting ROS and apoptosis

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Highlights

- Cisplatin down-regulates Apelin expression in HEI-OC1 cells and cochlear hair cells.
- Apelin protects HEI-OC1 cells and cochlear hair cells from cisplatin-induced injury *in vitro.*
- Apelin attenuates cisplatin-induced ROS and apoptosis in HEI-OC1 cells and hair cells *in vitro.*

Abstract

Apelin, a specific endogenous ligand of the G protein-coupled receptor APJ, suppresses oxidative stress and apoptosis *in vitro* and *in vivo*. The current study explored whether Apelin protects against toxicity induced by the anticancer drug cisplatin *in vitro*, and the possible mechanisms that underlie this protective effect. The results showed that Apelin was expressed in the mouse auditory cell line HEI-OC1 and in cochlear hair cells (HCs) and was significantly downregulated by cisplatin, whereas pre-treatment with exogenous Apelin significantly reduced cisplatin-induced apoptosis, and thus protected HEI-OC1 cells and cochlear HCs from cisplatin-induced injury. Furthermore, Apelin reduced reactive oxygen species (ROS) generation, rescued mitochondrial membrane potential disruption, inhibited JNK signaling and attenuated the expression of pro-apoptotic factors in HEI-OC1 cells and in cochlear explants treated with cisplatin. Our findings suggest that Apelin could be used as an

otoprotective agent for the prevention of cisplatin-induced ototoxicity.

Key words

Apelin; cisplatin; toxicity; ROS; apoptosis, hair cells

1. Introduction

Cisplatin is an anti-tumor drug commonly used in the treatment of clinical tumors due to its broad and efficient anti-tumor spectrum. However, the drug has ototoxic side effects, most seriously permanent hearing loss, for which no effective treatments are currently available[1]. It is widely accepted that cisplatin causes apoptosis of cochlear hair cells (HCs), causing ototoxicity to occur primarily in the cochlea in the inner ear[2,3]. Recent studies showed that excessive ROS production and mitochondrial dysfunction induced by cisplatin are key contributors to apoptosis in the cochlea[4]. In particular, excessive ROS production depletes the antioxidant defense mechanisms of cochlear HCs, disrupts mitochondrial function, interferes with cellular signaling pathways and triggers apoptotic cell death by activating caspase enzymes. However, the specific pathological mechanism(s) of cisplatin-induced ototoxicity remains unclear[5]. Inhibiting excessive ROS generation and apoptotic processes could be a strategy to prevent cisplatin-induced ototoxicity.

The bioactive peptide Apelin is a ligand of the G-protein coupled receptor APJ and is a key regulator of oxidative stress and apoptosis. The Apelin gene encodes an Apelin precursor peptide containing 77 amino acids, which can be hydrolyzed into

several C-terminal deletion variants including Apelin-12, Apelin-13, Apelin-17 and Apelin-36[6]. Apelin-13 is the major subtype that has the strongest biological activity, and is the variant used in the current study. Apelin is widely expressed in several tissues and organs such as heart, lung, brain, kidney, liver, blood vessels, gastrointestinal tract and adipose tissue[7,8]. Apelin exhibits protective effects in diseases that involve cellular injury due to its anti-oxidatitive and anti-apoptotic roles[9,10]. Apelin promotes the activity of the mitochondrial enzymes superoxide dismutase, catalase and glutathione peroxidase and reduces the formation of hydroxyl radicals and malondialdehyde, thus reducing renal ischemia/reperfusion damage[11]. [Pyr1]-apelin-13 reduces myocardial damage by decreasing oxidative injury in a rat model of myocardial infarction[12]. Apelin inhibits ROS-mediated DNA damage and up-regulates the MAPKs/Akt pathway to attenuate cisplatin-induced myocardial toxicity[13]. The peptide regulates apoptosis in several cell types and so has been suggested to be a potential target for therapeutics that could prevent apoptosis in certain diseases. In addition, Apelin has neuroprotective effects, which result from inhibition of apoptosis in ischemia-induced brain damage, intracerebral hemorrhage, Alzheimer's disease, and Parkinson’s disease[10,14-16]. Moreover, Apelin inhibits endoplasmic reticulum stress-induced apoptosis mediated by FoxO1, thus alleviating ischemia/reperfusion injury[17].

Mounting evidence shows that Apelin has anti-oxidative and anti-apoptotic roles in different cell types[11,12,15,18,19], but the role of Apelin in the cells of the inner ear has not yet been reported. Therefore, we aimed to determine the role of Apelin in

cisplatin-induced auditory cell damage, and the possible mechanism(s) underlying this process.

2. Materials and methods

2.1 HEI-OC1 culture and treatment

HEI-OC1 cells were cultured as described previously[20]. Cisplatin (P4394, Sigma-Aldrich) was used at a concentration of 30 μM because this concentration caused apoptosis and injury in HEI-OC1 cells and HCs. HEI-OC1 cells were pretreated with varying concentrations of Apelin (057-18, Phoenix Pharmaceuticals) for 2 h followed by cisplatin treatment. Cells were then used for the designated experiments.

2.2 Cochlear organ culture and treatment

C57BL/6 mice were sacrificed at four days old and the cochlear organs were dissected out quickly in cold Hank's buffer and pasted on small round coverslips. The cochlear explants were cultured in DMEM/F12 medium containing 10% fetal bovine serum. They were then treated with 30 μM cisplatin for 24 h, or pretreated with 10 nM Apelin for 2 h followed by cisplatin treatment for another 24 h. All animal experiments were performed according to the ordinance and ethical requirements of the Animal Care Committee of Jining Medical University.

2.3 Immunofluorescence staining

All specimens were fixed with 4% paraformaldehyde (PFA), washed with PBS, permeabilized with 1% Triton-X 100 in PBS for 10 min, blocked with 1% BSA for 1 h at room temperature, and then incubated with primary antibodies overnight at 4℃. Specimens were then incubated with the fluorescent secondary antibodies and DAPI

(1:1000, D9542, Sigma) for 1 h in the dark at room temperature. The specimens were analyzed using a confocal microscope (LEICA TCS SP8). The primary antibodies used in immunofluorescence staining were as the follows: rabbit anti-Apelin (1:800, ab59469, Abcam), rabbit anti-APJ (1:800, ab214369, Abcam), mouse anti-myosin7 α (1:1000, DSHB), and rabbit anti-cleaved caspase-3 (1:1000, 9664S, Cell Signaling Technology).

2.5 Cell viability assay

HEI-OC1 cells were seeded at a density of 8×10^3 cells/well in a 96-well plate and treated with 30 μM cisplatin for 24 h, or treated with 0.1 nM, 1 nM, 10 nM or 100 nM Apelin for 24 h, or pre-treated with Apelin for 2 h followed by cisplatin treatment for another 24 h. After the designate treatments, 10 μl CCK-8 (96992, Sigma-Aldrich) was added to each well and incubated for 2 h at 33℃. Cell viabilities were analyzed with an ELISA reader (Multiskan MK3). The cell viability of control group was taken as 100%. Three independent experiments were performed.

2.6 Protein electrophoresis separation and immunoblotting

Cells and cochlear explants were collected and lysed in RIPA buffer (P0013B, Beyotime Biotechnology) containing 1% protease inhibitor cocktail (P8340-1ML, Merck KGaA) for 30 min on ice. Equal amounts of proteins were loaded on a sodium dodecyl sulfate polyacrylamide gel and separated by electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes (PVDF; Millipore). PVDF membranes were blocked with 5 % milk in TBS buffer containing 0.05 % Tween20 (TBST) for 2 h and then incubated with the primary antibodies in 3 % milk in TBST

overnight at 4 °C followed by incubation with secondary antibodies for 1 h at room temperature. Protein signals were detected using an enhanced chemiluminescence (ECL) kit (WBULS0100, Millipore) and analyzed using ImageJ software (Broken Symmetry Software). The primary antibodies used were as follows; rabbit anti-cleaved caspase-3 (1:1000, 9664S, Cell Signaling Technology), mouse anti-cleaved caspase-9 (1:1000, Asp353, Cell Signaling Technology), rabbit anti-bax (1:1000, ab32503, Abcam), rabbit anti-phospho-JNK (1:500, 9251S, Cell Signaling Technology), mouse anti-total-JNK (1:1000, 9252S, Cell Signaling Technology), mouse anti-GAPDH (1:2000, TA-08, ZSGB-BIO), and mouse anti-β-actin (1:2000, TA-09, ZSGB-BIO).

2.7 TUNEL staining

A TUNEL kit (C10617, Life technologies) was used to detect apoptotic cells according to the manufacturer's protocols. First, samples were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature, incubated with TUNEL working solution for 1 h, and shielded from light at 37 °C. The nuclei were stained by DAPI for 30 min. The specimens were analyzed using a confocal laser microscope (LEICA TCS SP8).

2.8 Flow cytometry

An Annexin V-FITC Apoptosis Kit (BD Biosciences, CA) was used to detect apoptotic cells according to the manufacturer's protocols. HEI-OC1 cells were collected into 10 ml centrifuge tubes, centrifuged at 1000g, and then resuspended in 1X binding buffer. Annexin V-FITC (5 μL) and 10 μL PI were added to the cell

suspension and mixed gently, after which the cells were incubated at room temperature for 15 min in the dark. Samples were immediately subjected to flow cytometry using a FACS Calibur system (BD Biosciences) and the data were analyzed using Flow Jo7.6 software. Experiments were repeated three times.

2.9 ROS detection

HEI-OC1 cells and cochlear explants were incubated with 10 μM DCFH-DA (D6883, Sigma Technologies) or 5 μM Mitosox-red probes (1771410, Life Technologies) in serum-free DMEM for 30 min in the dark. After washing with PBS, the fluorescence signal was detected using a fluorescence microscope or FACS Calibur system.

2.10 Mitochondrial membrane potential detection

HEI-OC1 cells and cochlear explants were incubated with 1 μM Rhodamine 123 (C2007, Beyotime Biotechnology) in PBS for 30 min at 37°C in an incubator. They were then washed with PBS and the fluorescence signals were detected using a fluorescent microscope.

2.11 Statistical analysis

Data were presented as mean ± SD, and analyzed by GraphPad Prism 6 software using Student's t-test or one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Apelin and APJ are expressed in HEI-OC1 cells and mouse cochlear HCs

We first investigated whether auditory cells express Apelin and APJ, using HEI-OC1 cells, a conditionally immortalized cochlear cell line derived from mouse organ of Corti, and HCs labeled with Myosin7 α (a specific marker of HCs) in cultured cochlear explants. Results showed that Apelin and APJ were expressed in HEI-OC1 cells and HCs (Fig. 1A-D). In addition, the expression of APJ was significantly higher in outer hair cells (OHC) than in inner hair cells (IHC). However, there was no marked difference in the expression levels of Apelin in IHCs and OHCs. These results suggested that the Apelin–APJ axis might play a potential role in auditory cells, especially in OHCs, and provided a basis for further study.

(Fig.1)

3.2 Endogenous Apelin is down-regulated by cisplatin in HEI-OC1 cells and mouse cochlear HCs.

Endogenous Apelin expression was examined in HEI-OC1 cells at 0 h, 6 h, 12 h and 24 h after treatment with 30 μM cisplatin. Immunofluorescence staining showed that Apelin was downregulated by cisplatin in a time-dependent manner. Apelin expression was significantly decreased at 6 h and was lowest at 24 h (Fig. 2A). Down-regulation of Apelin by cisplatin was confirmed by western blot analysis (Fig. 2B). The expression of Apelin was also detected in HCs treated with or without cisplatin for 24 h. Immunofluorescence data showed that Apelin expression was significantly

lower in surviving OHCs and IHCs treated with cisplatin for 24 h than in the control group (Fig. 2C). Down-regulation of Apelin was confirmed by western blot analysis in cochlear explants treated with cisplatin for 24 h (Fig. 2D).

(Fig.2)

3.3 Apelin protects HEI-OC1 and cochlear HCs from cisplatin-induced injury.

Data from the CCK-8 assay showed that Apelin alone had no effect on HEI-OC1 cell viability, but significantly increased the viability of HEI-OC1 cells treated with cisplatin in a concentration-dependent manner. The protective effect of 10 nM Apelin was greater than that of 1 nM Apelin in cisplatin-injured cells, but was not significantly different from that of 100 nM cisplatin. Therefore, 10 nM was selected for subsequent experiments (Fig. 3A). As shown in Fig. 3B, Apelin-treated and control OHCs (three rows) and IHCs (one row) were of normal shape and were arranged neatly. Conversely, cisplatin treatment resulted in degeneration and a disordered arrangement of HCs. This was accompanied by loss of HCs, which appeared aggravated gradually from apex turns to basal turns. Apelin pretreatment significantly alleviated cisplatin-induced HC damage (Fig. 3B). Total HC counts (including IHCs and OHCs) showed that HC loss was more severe in basal and middle turns of cisplatin-treated cochlear explants than that in control explants, whereas Apelin pre-treatment significantly attenuated cisplatin-induced HCs loss in the basal and middle turns (Fig. 3C-E). However, the total number of HCs in the apex turns had no significant difference between cisplatin- and Apelin plus cisplatin-treated explants. These results indicate that Apelin plays a protective role in cisplatin-treated

HEI-C1 cells and HCs of cochlear explants, especially in the middle and basal turns. (Fig.3)

3.4 Apelin prevents cisplatin-induced apoptosis in HEI-OC1 cells.

Apoptotic cells were examined using flow cytometry and TUNEL staining. Flow cytometry results showed that the proportion of apoptotic cells was significantly higher in the cisplatin-treated group than in the control group; this effect was decreased by 10nM Apelin pretreatment (Fig. 4A-B). The number of TUNEL-positive stained apoptotic cells was significantly higher in cisplatin-treated group, but was significantly decreased by 10nM Apelin pretreatment (Fig. 4C-D). These results indicated that Apelin prevented cisplatin-induced apoptosis in HEI-OC1 cells. (Fig.4)

3.5 Apelin alleviates cisplatin-induced apoptosis in HCs of cochlear explants.

The number of TUNEL-positive stained cells was significantly higher in cochlear explants treated with cisplatin than that in the control group; Apelin pretreatment significantly decreased the number of stained cells (Fig. 5A-B). Furthermore, the number of cisplatin-induced apoptotic HCs co-stained by TUNEL and myosin7 α was also significantly decreased by Apelin pretreatment (Fig. 5A, 5C). Similarly, Apelin pretreatment significantly decreased the number of cleaved caspase3-positive stained cells as well as the number of cleaved caspase-3/Myosin7a co-stained cells in cochleae treated with cisplatin (Fig. 5D-F). These results implied that Apelin protected HCs from cisplatin-induced damage by inhibiting apoptosis. (Fig.5)

3.6 Apelin diminishes cisplatin-induced ROS and disruption of mitochondrial membrane potential in HEI-OC1 cells and cochlear explants.

DCFH-DA and Mitosox-red probes were used to monitor ROS generation in HEI-OC1 cells and cochlear explants treated with cisplatin or cisplatin combined with Apelin. Immunofluorescence and flow cytometry results showed that fluorescence signals of DCFH-DA and Mitosox-red were highest in HEI-OC1 cells treated with cisplatin, and were lower in cells pre-treatment with Apelin, indicating that Apelin suppressed cisplatin-induced ROS generation in HEI-OC1 cells (Fig. 6A-D). Similarly, Apelin also significantly reduced the cisplatin-induced increase in DCFH-DA and Mitosox-red fluorescence signals in cochlear explants (Fig. 6E-F). Taken together, these results indicate that Apelin inhibited cisplatin-induced excessive ROS production, which is known to potentiate cisplatin-induced ototoxicity. In addition, a Rhodamine 123 probe was used to detect the mitochondrial membrane potential of HEI-OC1 cells and cochlear cells. Rhodamine 123 fluorescence was higher in HEI-OC1 cells and cochlear explants treated with cisplatin than in control cells, but was reduced by Apelin pre-treatment (Fig. 6G-H). These results indicate that Apelin prevented the disruption in mitochondrial membrane potential caused by cisplatin in HEI-OC1 cells and cochlear explants *in vitro.*

(Fig.6)

3.7 Apelin inhibits cisplatin-induced activation of JNK signaling and caspase-dependent apoptosis in HEI- OC1 cells and cochlear explants.

To elucidate the mechanisms underlying the protective effects of Apelin on cisplatin-

induced toxicity *in vitro*, JNK signaling and the levels of apoptotic-related factors, including cleaved caspase-3, cleaved caspase-9 and bax, were examined. We found that Apelin pre-treatment significantly reduced the cisplatin-induced increase in p-JNK protein levels (Fig. 7A-B) and reduced the cisplatin-induced increase in cleaved caspase-3, cleaved caspase-9 and bax levels in HEI-OC1 cells (Fig. 7C-F). In addition, the expression of p-JNK, cleaved caspase-3, cleaved caspase-9 and bax was assayed by western blot in cochlear explants treated with cisplatin or cisplatin combined with Apelin (Fig. 7H-L). These results indicated that Apelin protect against cisplatin-induced toxicity through inhibition of JNK signaling and caspase-dependent apoptosis in HEI-OC1 cells and cochlear explants.

(Fig.7)

4. Discussion

Prior to this study it was unknown whether the widely expressed bioactive peptide Apelin and its cognate receptor are expressed in auditory cells. We initially showed that Apelin and APJ are expressed in HEI-OC1 cells and cochlear HCs from mice. This result widens our knowledge of expression range of Apelin and APJ in mammals, and provides a foundation for further evaluation of the potential role of Apelin in cisplatin-induced ototoxicity.

Apelin has protective roles in, and is down-regulated in several diseases such as ischemia-reperfusion injury, neurodegenerative diseases, metabolic disorders and aging[21,22]. In this study, endogenous Apelin was down-regulated by cisplatin in HEI-OC1 cells and cochlear explants *in vitro*. These results indicated that down-regulation of Apelin expression may be related to cisplatin-induced damage to inner ear cells. We hypothesized that exogenous Apelin pretreatment could alleviate cisplatin-induced damage. Indeed, exogenous Apelin protected HEI-OC1 cells and HCs from cisplatin-induced injury, while Apelin itself had no cytotoxic effect on these cells. Moreover, the protective effect of Apelin was greater in HCs in basal and middle turns than in apex turns.

It is well established that cisplatin-induced apoptosis eventually leads to HC loss and hearing impairment[3]. In this work, we found that exogenous Apelin pretreatment alleviated cisplatin-induced apoptosis in HEI-OC1 cells and HCs *in vitro*, implying that Apelin protected against cisplatin-induced ototoxicity via inhibition of apoptosis.

These results are consistent with studies demonstrating that Apelin has anti-apoptotic effects in other diseases such as ischemia-induced brain damage, intracerebral hemorrhage, Alzheimer's disease, and Parkinson's disease[14,15,17,23]. Taken together, our results suggest that Apelin provides otoprotection against cisplatin-induced injury *in vitro* by inhibiting apoptosis.

Exposure to high doses of cisplatin leads to excessive ROS accumulation in cochlea[24]. Excessive ROS production can cause mitochondrial dysfunction in HCs, leading to apoptosis[25]. Application of antioxidants is an effective protective strategy to reduce cisplatin-induced ototoxicity[26,27]. Apelin improves the function of mitochondria and decreases ROS production in oxidative stress-induced apoptosis. In addition, Apelin prevents ROS-dependent hypertrophy by enhancing antioxidant activity in the heart, and structural analogs of Apelin inhibit mitochondria-derived ROS production and apoptosis in cortical neurons[9,14]. Apelin also attenuated oxidative stress in human adipocytes[28]. In this work, we showed that Apelin significantly reduced cisplatin-induced increases in intracellular ROS in both HEI-OC1 cells and cochlear explants. This observation is consistent with a report demonstrating Apelin-13 protects hair cell-like cells treated with by hydrogen peroxide (H_2O_2) from oxidative stress-induced apoptosis[29]. Therefore, we suggest that the otoprotective effect of Apelin is closely associated with its ability to inhibit excessive ROS production, which is known to potentiate cisplatin-induced apoptosis. Excessive ROS production is associated with disruption of mitochondrial membrane potential, which leads to interruption of the mitochondrial electron transfer chain and

mitochondrial dysfunction[30]. The dysfunction of mitochondria in turn potentiates excessive ROS generation and apoptosis. In this study, Apelin significantly extenuated the cisplatin-induced loss of mitochondrial membrane potential in HEI-OC1 and cochlear explants, implying that Apelin rescued mitochondrial dysfunction.

ROS–JNK signaling is involved in cochlear cell apoptosis induced by a variety of stimuli, and might be a promising therapeutic target for treating hearing impairment[31]. Our results showed that Apelin inhibited cisplatin-induced activation of JNK signaling both in HEI-OC1 cells and cochlear explants, implying that Apelin might alleviate cisplatin-induced apoptosis by inhibiting JNK signaling. It is well known that bax, caspase-9, and caspase-3 are involved in the apoptotic pathway. Bax increases mitochondrial membrane permeability, leading to activation of caspase-9 and caspase-3, thereby initiating activation of apoptotic pathways. In this work, Apelin significantly reduced the cisplatin-induced increase in expression of pro-apoptotic factors including bax, cleaved caspase-9 and cleaved caspase-3 in HEI-OC1 cells and cochlear explants *in vitro*. These results indicate that Apelin may prevent cisplatin-induced ototoxicity by inhibiting the caspase-dependent apoptotic pathway.

In conclusion, we have shown that that Apelin protects against cisplatin-induced toxicity by reducing ROS generation, inhibiting JNK signaling, and attenuating apoptosis *in vitro*. Our findings indicate that Apelin could be an effective agent for preventing cisplatin-induced damage in auditory cells. Because this study was performed in vitro, further studies will be required to determine whether Apelin protects against cisplatin-elicited toxicity in the inner ear in vivo.

Credit Author Statement

Haiyan Yin: Conceptualization, Methodology, Investigation, Formal Analysis, Writing -Original Draft, Writing, review & editing

Hui Zhang: Methodology, Investigation, Formal Analysis, Writing-Original Draft

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Yan Guo, Yang Gao and Lili Yuan: Investigation, Formal Analysis

Xinxin Yang: Conceptualization, Formal Analysis, Writing, review & editing

Jing Chen: Supervision, Funding Acquisition

Competing interests

All authors declare no conflicts of interest.

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Figure legends

Figure 1 Apelin and APJ are expressed in HEI-OC1 cell and mouse cochlear HCs

(A-B) Immunofluorescence staining of Apelin (red staining) and APJ (red staining) expression in HEI-OC1 cells. (C-D) Immunofluorescence staining of Apelin (red staining) and APJ (red staining) expression in cochlear HCs labeled with Myosin7a (green, outer hair cell; OHC, inner hair cell; IHC, white arrows). Scale bar = 10 μm.

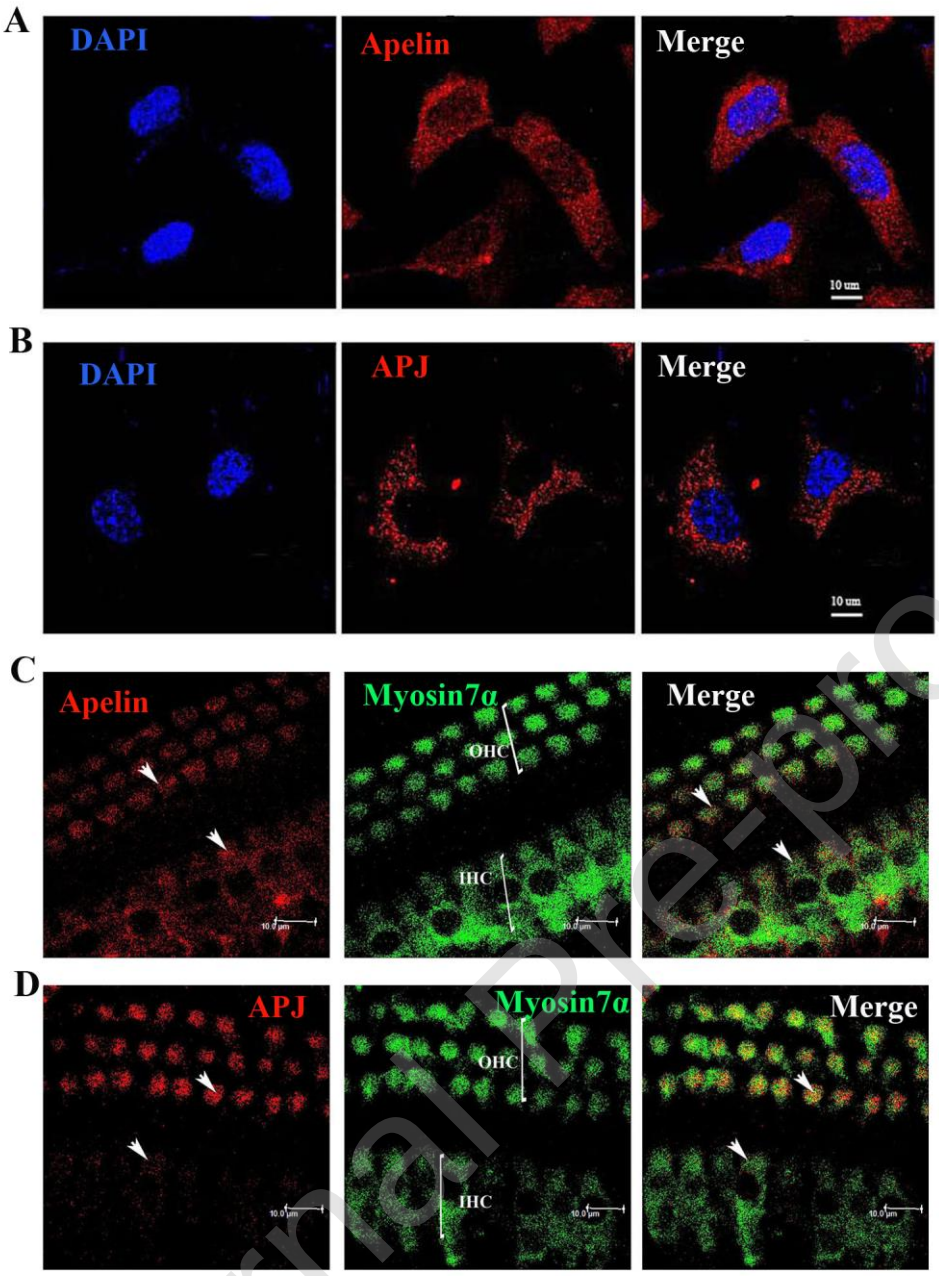

Figure 2 Endogenous Apelin expression is downregulated by cisplatin in HEI-OC1 cells and mouse cochlear HCs *in vitro*

(A) Representative immunofluorescence staining of Apelin (red staining)

expression in HEI-OC1 cells exposed to 30 μM cisplatin for various times. (B) Apelin expression was assayed by western blot in HEI-OC1 cells exposed to 30 μM cisplatin for various times. (C) Immunofluorescence staining of Apelin (red staining) expression in HCs labeled by Myosin7α (green staining) in middle turns of cochlear explants treated with 30 μM cisplatin for 24 h. (D) Apelin expression was assayed by western blot in cochlear explants treated with 30 μM cisplatin for 24 h. (*$P < 0.05$, **$P < 0.01$). n = 3. Scale bar = 25 μm.

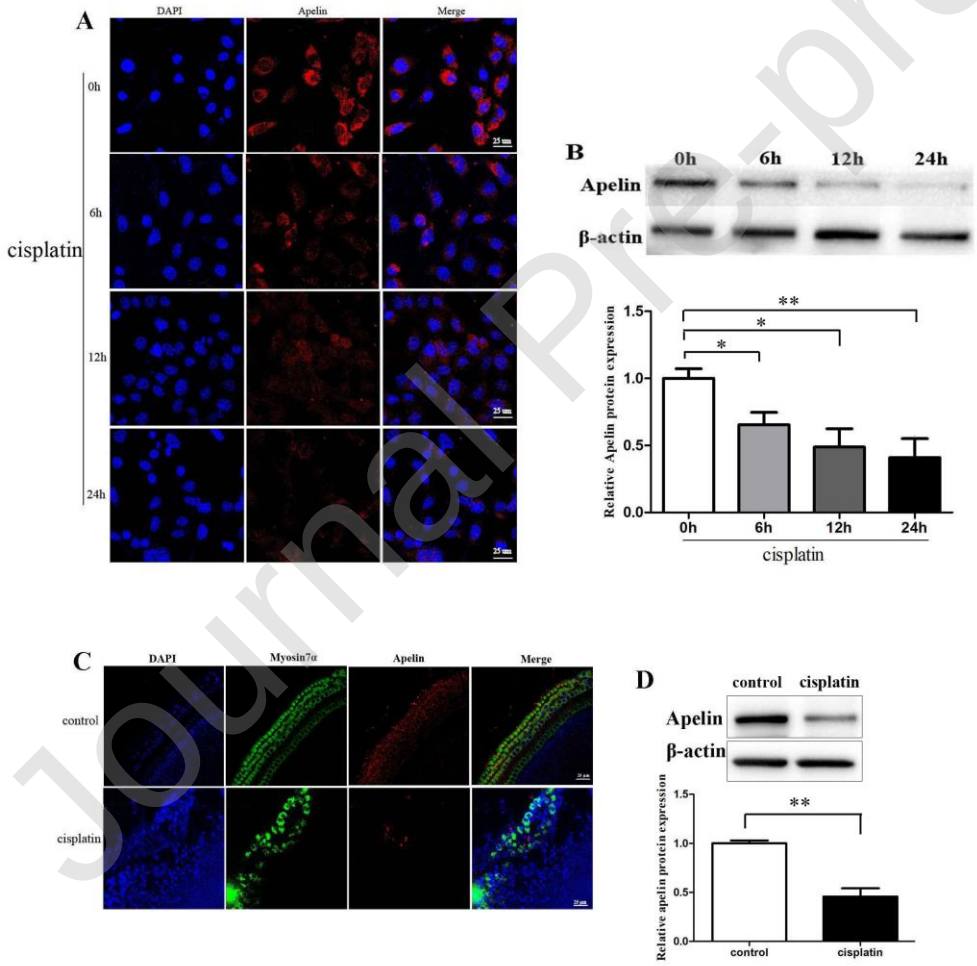

Figure 3 Apelin alleviates cisplatin-induced injury in HEI-OC1 cells and mouse

cochlear HCs

(A) Cell viabilities were measured using a CCK-8 assay in cells treated with various concentrations and combinations of cisplatin and Apelin (*$*P < 0.05$*, **$**P < 0.01$*, $^{ns}P > 0.05$), n=6. (B) Immunofluorescence staining of HCs labeled with Myosin 7a (green staining) in apical, middle and basal turns in control, Apelin-, cisplatin- and Apelin plus cisplatin-treated explants groups. (C-E) Total survival HCs counts in apical, middle and basal turns in control, Apelin-, cisplatin- and Apelin plus cisplatin-treated explants. (*$*P < 0.05$*, $^{ns}P> 0.05$). n=3.

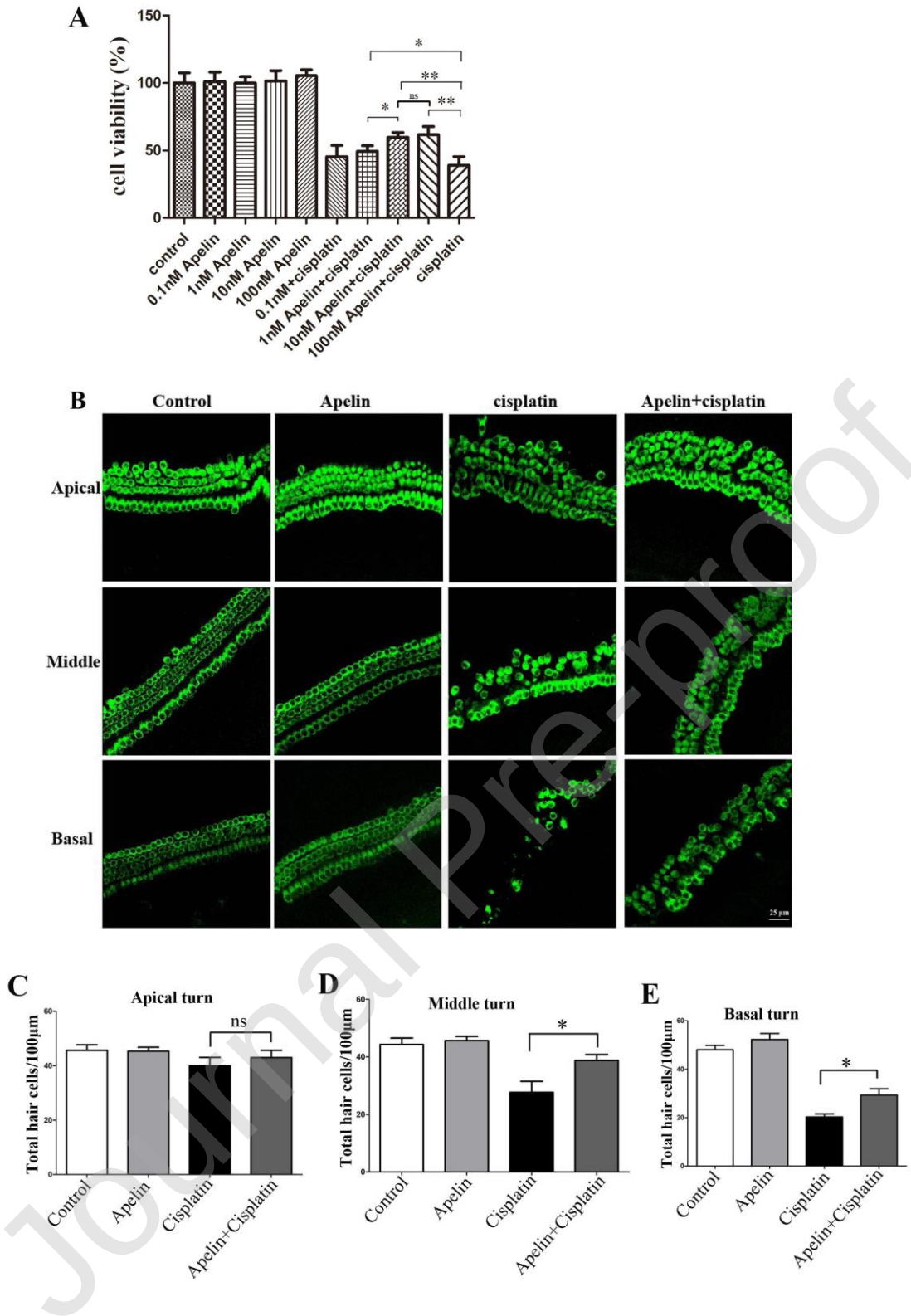

Figure 4 Apelin reduces cisplatin-induced apoptosis in HEI-OC1 cells

(A) Apoptotic cells were analyzed using flow cytometry in control, cisplatin- and

Apelin plus cisplatin-treated cells. (B) Quantification of the proportion of apoptotic cells (gate%) in control, cisplatin- and Apelin plus cisplatin-treated cells. (C) TUNEL staining (green) in control, cisplatin- and apelin plus cisplatin-treated cells. (D) Quantification of the percentage of TUNEL-positive cells in different groups. (*$p< 0.05$, **$p< 0.01$). n=3.

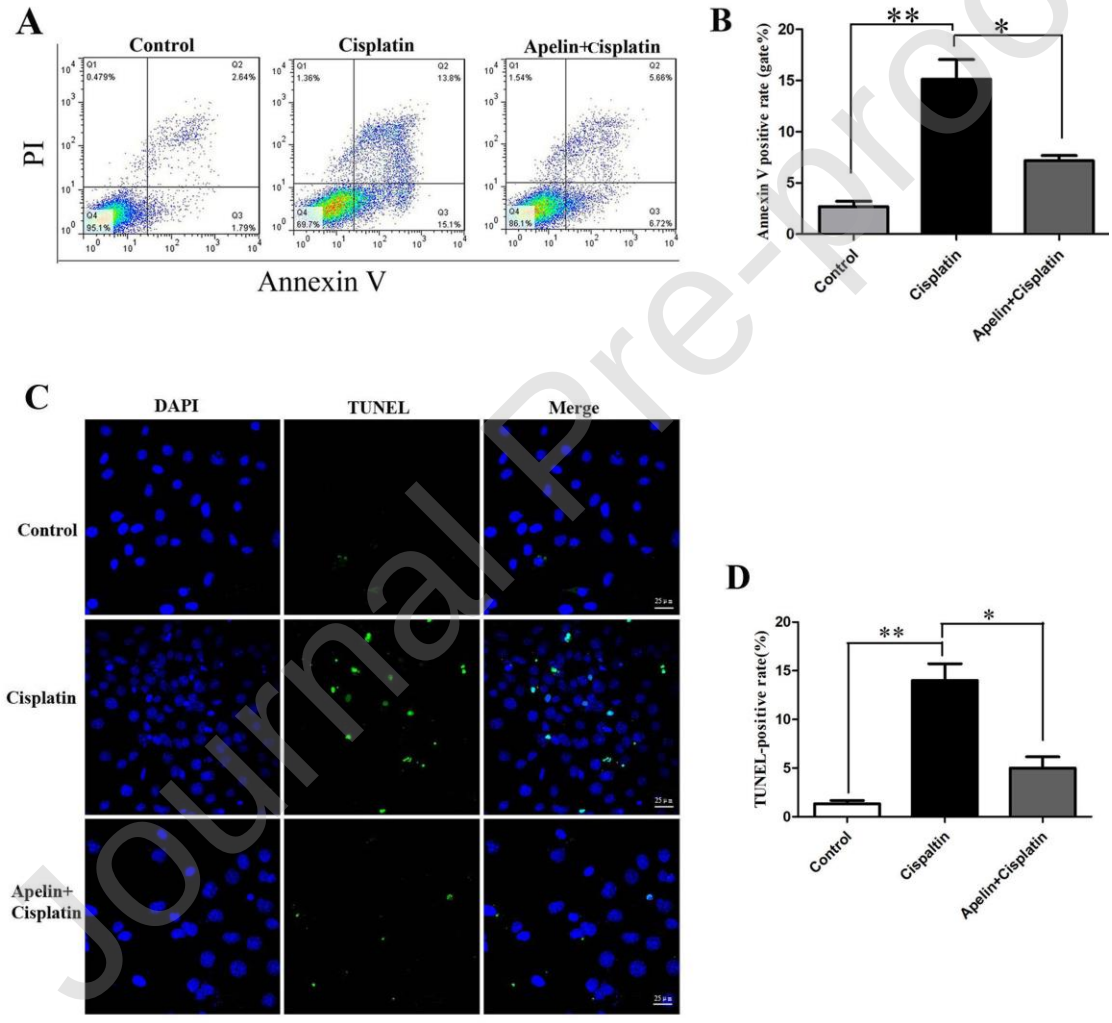

Figure 5 Apelin protects mouse cochlear HCs from cisplatin-induced apoptosis

(A) Immunofluorescence of TUNEL staining in the middle turns of cochlear

explants in control, cisplatin- and Apelin- plus cisplatin-treated explants. (B) Quantification of TUNEL-positive cells in the middle turns of cochlear explants in control, cisplatin- and Apelin plus cisplatin-treated explants. (C) Quantification of apoptotic HCs co-stained by TUNEL/Myosin α in control, cisplatin- and Apelin plus cisplatin-treated explants. (D) Immunofluorescence staining of cleaved caspase-3 in the middle turns of control, cisplatin- and Apelin plus cisplatin-treated explants. (E) Quantification of cleaved caspase-3-positive cells in control, cisplatin- and Apelin plus cisplatin-treated explants. (F) Quantification of apoptotic HCs co-stained by cleaved caspase-3/Myosin7 α in control, cisplatin- and Apelin plus cisplatin-treated explants. (**$p<0.01$). n=3.

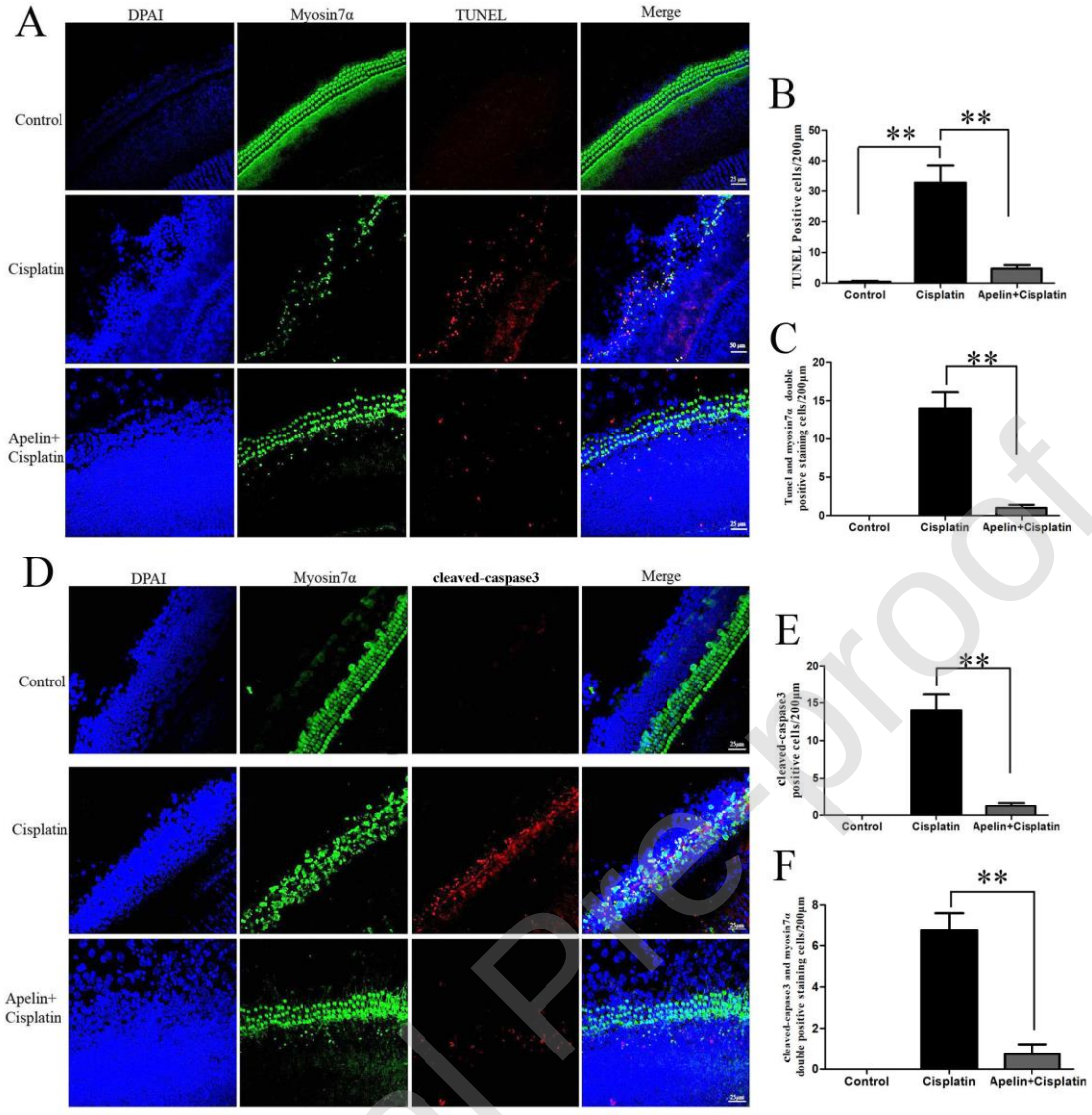

Figure 6 Apelin attenuates cisplatin-induced ROS and dysfunction of mitochondrial membrane potential in HEI-OC1 cells and cochlear explants

(A) DCFH-DA staining, (B) ROS generation monitored by DCFH-DA and assayed by flow cytometry, (C) Mitosox-red staining and (D) ROS generation monitored by Mitosox-red assayed by flow cytometry in HEI-OC1 cells of control, cisplatin- and Apelin plus cisplatin-treated groups. (E) DCFH-DA staining and (F) Mitosox-red staining in cochlear explants of control, cisplatin- and Apelin plus cisplatin-treated groups. (G-H) Rhodamine 123 staining in HEI-OC1 cells

and cochlear explants of control, cisplatin- and Apelin plus cisplatin-treated groups.

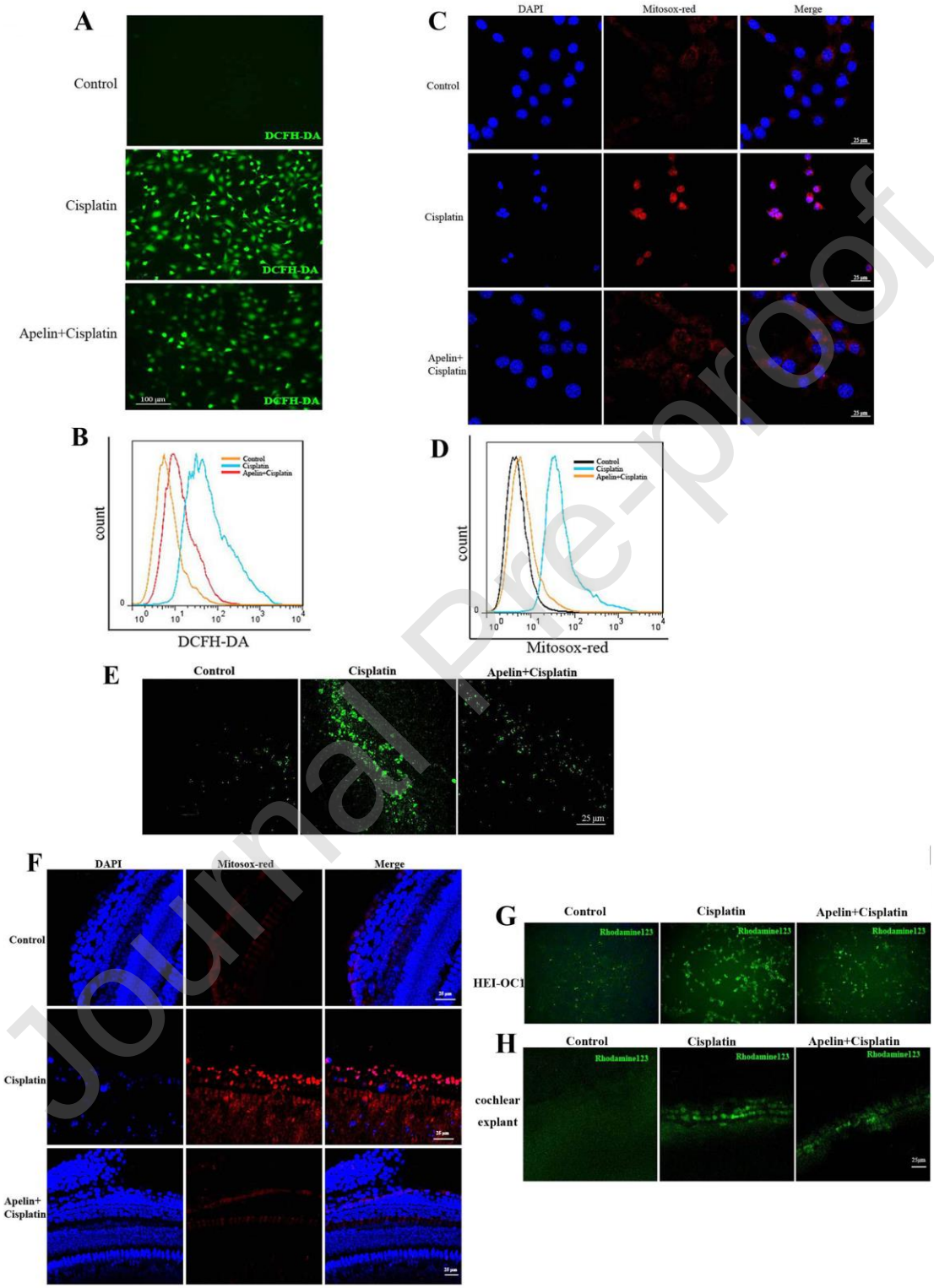

Figure 7 Apelin inhibits cisplatin-induced JNK signaling and caspase-dependent apoptosis in HEI-OC1 cells and cochlear explants

(A, B) p-JNK levels were detected by western blot and analyzed in control, Apelin plus cisplatin- and cisplatin-treated HEI-OC1 cells. (C, D, E, F) The levels of cleaved caspase-3, cleaved caspase-9 and bax proteins were detected by western blot and analyzed in control, Apelin plus cisplatin- and cisplatin-treated HEI-OC1 cells. (G, H) The levels of p-JNK protein were determined by western blot and analyzed in control, Apelin plus cisplatin-treated and cisplatin-treated cochlear explants. (I, J, K, L) The levels of cleaved caspase-3, cleaved caspase-9 and bax proteins were analyzed by western blot in control, Apelin plus cisplatin- and cisplatin-treated cochlear explants. GAPDH and β -actin served as a loading control. (*$p < 0.05$, **$p < 0.01$). n=3.