Panax notoginseng Saponins protect auditory cells against cisplatin-induced ototoxicity by inducing the AKT/Nrf2 signaling-mediated redox pathway

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Abstract. Cisplatin-induced cytotoxicity, such as nephrotoxicity, neurotoxicity and ototoxicity, restricts the clinical application of this compound. *Panax notoginseng* Saponins (PNS) exhibit potent free radical scavenging and antioxidant activity. PNS have been demonstrated to reduce cisplatin-induced nephrotoxicity and neurotoxicity. The present study investigated the ability of PNS to protect the auditory HEI-OC1 cell line against ototoxicity induced by cisplatin. PNS induced activation of the AKT/nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Following pretreatment with PNS, HEI-OC1 cells were treated with cisplatin and cultured for 24 h. The viability of HEI-OC1 cells was examined using a Cell Counting Kit-8 assay. Double staining analysis was used to measure cell apoptosis. The ability of PNS to reduce reactive oxygen species (ROS) levels was assessed by flow cytometry. The levels of phosphorylated (p)-AKT, heme oxygenase 1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), glutamate-cysteine ligase catalytic (GCLC) and Nrf2 were measured by western blotting. HEI-OC1 cells that were pretreated with PNS exhibited significantly increased cell viability compared with that noted in cells treated only with cisplatin. In addition, PNS suppressed the induction of apoptosis and ROS production following cisplatin treatment. The upregulation of NQO1, HO-1 and GCLC expression in PNS-pretreated cells was associated with p-AKT levels and the activation of Nrf2. These findings suggested that PNS protected auditory cells against ototoxicity induced by cisplatin by activating AKT/Nrf2 signaling. PNS may serve as a potential candidate in regulating cisplatin-induced cytotoxicity.

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Introduction

Cisplatin is a typical chemotherapeutic drug that is widely used in the treatment of a variety of solid tumors (1,2). However, the dose-limiting side effects of high concentrations of cisplatin, such as renal damage, neurotoxicity and ototoxicity can restrict its clinical application (3-5). Among patients that receive cisplatin chemotherapy, >90% may end up with ear-associated adverse effects (3,6). However, currently no effective treatment exists to avoid or reduce ototoxicity induced by cisplatin (5,7-9). Therefore, the prevention of ototoxicity induced by cisplatin has become a major focus in tumor therapy.

To the best of our knowledge, the mechanism of the aforementioned process has not yet been elucidated. However, additional evidence has demonstrated that overproduction of reactive oxygen species (ROS) serves a key role in this process (10,11). High accumulation of cisplatin in cochlear tissues may reduce the expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2), a well-known transcription factor responsible for the antioxidant-pro-oxidant balance (12,13). This is caused by modulation of the expression levels of various antioxidant enzymes, including heme oxygenase 1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1) and glutamate-cysteine ligase catalytic (GCLC) (14-16). This pathological process disrupts the balance between ROS production, and antioxidant enzyme activity and expression, leading to auditory cell injury and apoptosis (17). Therefore, upregulation of Nrf2 expression and the increase in antioxidant enzyme expression has become an important target for treating cisplatin-induced ototoxicity.

Panax notoginseng Saponins (PNS) are compounds that are extracted from *Panax notoginseng* (Sanqi).

Panax notoginseng is routinely used to treat acute cerebral infarction and acute myocardial infarction (18,19). A number of previous reports have demonstrated that PNS can inhibit cisplatin-induced nephrotoxicity and neurotoxicity (20-23). However, whether PNS can inhibit cisplatin-induced ototoxicity remains unknown. A previous study indicated that PNS could regulate Nrf2 antioxidant signaling via the AKT signaling pathway (24). Therefore, the present study examined whether PNS could protect against ototoxicity induced by cisplatin via activation of the AKT/Nrf2 signaling pathway. HEI-OC1 cells were incubated with cisplatin with or without pretreatment of PNS and subsequently cell viability and apoptotic rate were determined. Additionally, the associated signaling mechanisms were examined.

Materials and methods

Chemicals and materials. PNS was obtained from Chengdu Manst Biotechnology Co., Ltd. (www.cdmust.com). and dissolved in DMSO (cat. no. A1222; purity ≥98%). House Ear Institute-Organ of Corti 1 (HEI-OC1) cell lines derived from the auditory organ of a transgenic mouse were obtained from House Ear Institute. The antibodies for active caspase 3, Nrf2, NQO1 and Bcl-2 were obtained from Abcam. The antibodies for HO-1, GCLC, AKT, and phosphorylated-AKT (p-AKT) were obtained from Abcam. The Hoechst 33258 staining kit was obtained from Biyuntian Biotechnology Co., Ltd. Cisplatin and the propidium iodide (PI) staining kit were obtained from Sigma-Aldrich; Merck KGaA. The oxygen species assay kit was purchased from Nanjing KeyGen Biotech Co., Ltd. The p-AKT inhibitor LY294002 was purchased from Selleck Chemicals. Cells were treated with 25 μmol/l LY294002 for 24 h at room temperature before transfection. The culture media were obtained from Gibco; Thermo Fisher Scientific, Inc.

Cell culture and cell viability assay. The HEI-OC1 cell line is a frequently used model of ototoxicity assessment and expresses several molecular characteristics of Corti sensory cells (25,26). This cell line was selected for use in the present study. The cells were cultivated in complete medium containing high-glucose DMEM (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 10% FBS (Beijing Solarbio Science & Technology Co., Ltd.) and 50 U/ml interferon-γ without antibiotics. Cells were cultured at 33˚C in the presence of 10% CO_2 in a humidified incubator (permissive conditions). Cell toxicity was assessed as follows: A total of $5x10^3$ HEI-OC1 cells/well in 100 μl complete medium were seeded in a 96-well plate and cultured at 33˚C in the presence of 10% CO_2. The cells were pretreated with different concentrations of PNS (0, 25, 50, 100, 200, 400 and 800 μg/ml) for 2 h at room temperature. Subsequently, 20 μM cisplatin was added to each well of the plate. Following a 24-h cultivation, 10 μl Cell Counting Kit-8 (CCK-8; cat. no. CK-4; Dojindo Molecular Technologies, Inc.) solution was added to each well and incubated for an additional 30 min, according to the manufacturer's protocol. A microplate reader (Lab Systems Multiskan Ascent 354; MTX Lab Systems, Inc.) was used to detect cell viability at 450 nm. Finally, the appropriate doses of PNS were selected for further *in vitro* studies depending on the initial toxicity data.

Hoechst 33258 and PI double staining. A total of $1x10^5$ HEI-OC1 cells were diluted in 4 ml complete medium and incubated in 6-well plates with 100 μg/ml PNS for 2 h prior to treatment with cisplatin (20 μM) at 33˚C in a humidified 10% CO_2 environment for 24 h. Following two washes with PBS, the cells were fixed with 4% paraformaldehyde for 30 min at 4˚C, and stained with 2 μg/ml Hoechst 33258 and 1 μg/ml PI for 20 min in the dark at 4˚C. Finally, following two washes with PBS, the cells were visualized using a Leica DMi8 fluorescence microscope (magnification, x200; Leica Microsystems, Inc.). The highest emission was recorded at ~460 nm for PI staining and at 485 nm for Hoechst dyes. The amount of dead cells was quantified using ImageJ software version 1.8.0 (National Institutes of Health) and calculated as follows: Amount of Hoechst-positive cells/amount of PI-positive cells.

TUNEL staining. To assess the DNA fragmentation using TUNEL, cells were fixed with 1% formaldehyde for 10 min at room temperature. Following incubated with 100 μg/ml PNS for 2 h prior to treatment with cisplatin (20 μM) at 33˚C in a humidified 10% CO_2 environment for 24 h, a total of $1x10^5$ HEI-OC1 cells were incubated with 20 μg/ml proteinase K for 60 min at room temperature. The slides were then rinsed with PBS for 3 min, dried and incubated in 20 μl TUNEL reaction mixture for 1 h at room temperature. Subsequently, cells were treated with 2% H_2O_2 for 30 min at room temperature. After washing with PBS, sections were incubated with 150 U/ml anti-digoxigenin peroxidase conjugate for another 30 min at room temperature in a humidified atmosphere. Finally, sections were stained with 10 μg/ml diaminobenzene in the dark for 10 min at room temperature and counterstained with 20 μg/ml hematoxylin at room temperature for 10 min. Cell nuclei were stained with 0.1 μg/ml DAPI at 37˚C for another 10 min. After being washed with PBS for 3 min, 1% ammonia (cat. no. SJ00815F17008; Sigma-Aldrich; Merck KGaA), and rehydrated using different concentration of alcohol, the sections were sealed and detected with a fluorescent microscope (magnification, x200) with VECTASHIELD mounting media (Vector Laboratories, inc.) for fluorescence microscope. TUNEL positive (apoptotic) cells were quantified by counting amber-colored cells in 10 fields (6,000 μm^2/field). The specimens were visualized using a Leica confocal laser scanning microscope and processed with Photoshop CS6 (Adobe Systems, Inc.).

Determination of intracellular ROS. A total of $1x10^5$ HEI-OC1 cells were diluted in 4 ml complete medium and cultured in 6-well plates. The cells were pre-incubated with 100 μg/ml PNS for 2 h prior to incubation with cisplatin (20 μM) at 33˚C in a humidified 10% CO_2 environment for 24 h. Subsequently, the cells were washed with cold PBS twice and incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA; Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at room temperature. The cells were harvested into 1.5 ml tubes using trypsin-EDTA and washed with PBS twice.

Flow cytometry analysis. The quantification of cell death was determined via flow cytometry using the Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol (BD Pharmingen; BD Bioscience) on a BD

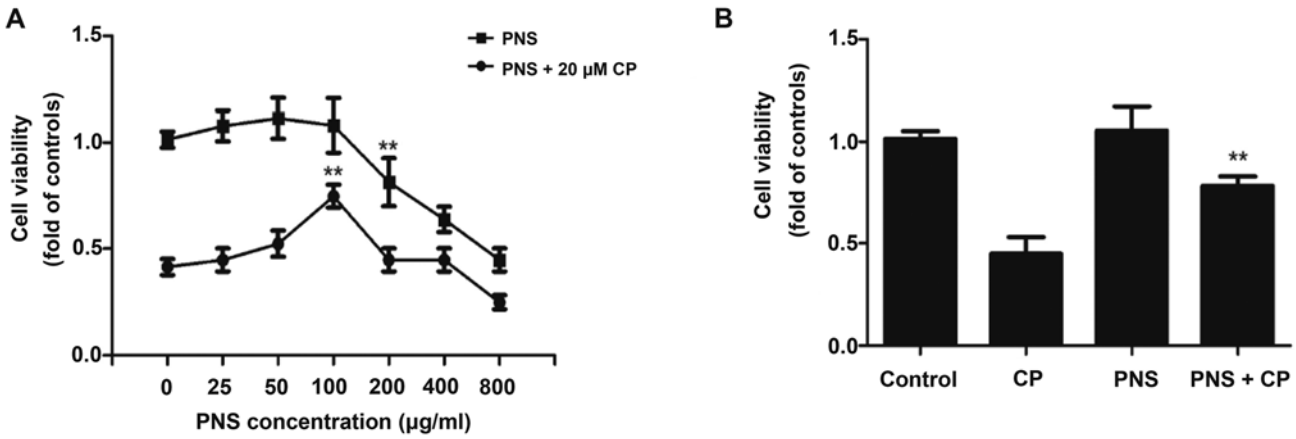

Figure 1. PNS prevents CP-induced cytotoxicity. (A) HEI-OC1 cell viability was determined following administration of various concentrations of PNS with or without 20 μM CP to the cells. (B) HEI-OC1 cell viability following treatment with 100 μg/ml PNS and 20 μM CP (n=3 per group). All data are presented as the mean ± SEM. **P<0.01 vs. CP group. CP, cisplatin; PNS, *Panax notoginseng* Saponins.

FACSCalibur flow cytometer (BD Biosciences) at an excitation and emission wavelength of 488 and 525 nm, respectively. Briefly, (10,000 events per sample) DCFH-DA-treated cells were seeded into each Petri dish (size, 30 mm) and after a 24 h incubation, various concentrations of the test compound (20 μM CP, 100 μg/ml PNS or 20 μM CP + 100 μg/ml PNS) were added and incubated for 24 , 48 and 72 h at room temperature. The cells were washed with PBS, suspended in Annexin V binding buffer and then added to an Annexin V-FITC solution and propidium iodide (PI) for 10 min at room temperature. The percentage of apoptotic cells was calculated using CellQuest software version 5.1 (Becton, Dickinson and Company). Data are presented as the percentage of Annexin V-stained cells, and all experiments were performed in triplicate. Morphological changes were imaged under a phase contrast microscope (magnification, x200; Olympus Corporation).

Western blot analysis. Total protein was extracted from cells using the Cell Total Protein Extraction kit (Sigma-Aldrich; Merck KGaA). The concentration of the proteins was measured using the bicinchoninic acid method. The 20 μg/lane protein samples were separated by 10% SDS-PAGE and analyzed by electrophoresis. The proteins were transferred to PVDF membranes and blocked in 3% BSA (Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Subsequently, the membranes were incubated at 4˚C overnight with the following primary antibodies: Active caspase-3 (dilution, 1:2,000; rabbit; cat. no. ab214430; Abcam), Bcl-2 (dilution, 1:1,000; rabbit; cat. no. ab182858; Abcam), GCLC (dilution, 1:2,000; rabbit; cat. no. ab190685; Abcam), NQO1 (dilution, 1:2,000; rabbit; cat. no. ab80588; Abcam), HO-1 (dilution, 1:10,000; rabbit; cat. no. ab68477; Abcam), Nrf2 (dilution, 1:2,000; rabbit; cat. no. ab62352; Abcam), AKT (dilution, 1:2,000; rabbit; cat. no. ab179463; Abcam), p-AKT (dilution, 1:2,000; rabbit; cat. no. ab192623; Abcam) or GAPDH (dilution, 1:3,000; rabbit; cat. no. ab181602; Abcam). The next morning, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase IgG (dilution, 1:2,000; cat. no. ab181658; Abcam) for 1 h. Finally, the proteins were visualized using the WesternBright™ ECL kit (cat. no. E-IR-R301; Elabscience, Inc.) and analyzed using ImageJ software version 1.8.0 (National Institutes of Health).

Statistical analysis. SPSS v22.0 software (IBM Corp.) was used for the statistical analysis and GraphPad Prism 6 software (GraphPad Software, Inc.) was used for the generation of statistical charts. All data are presented as the mean ± standard error of the mean. All the experiments in this study were conducted in triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

PNS prevents cisplatin-induced cytotoxicity. As shown in Fig. 1A, at concentrations up to 100 μg/ml PNS, the cell viability of HEI-OC1 was not significantly affected. However, there was a significant variation between 100 and 200 μg/ml PNS. The results of the cell viability assay indicated that in the presence or absence of cisplatin, the viability of HEI-OC1 cells was not affected by pretreatment with PNS concentrations <100 μg/ml. Therefore, 100 μg/ml PNS could be used in the subsequent experiments in order to avoid toxicity. Furthermore, after treated with 20 μM CP, it was found that 100 μg/ml PNS was able to prevent cisplatin-induced cytotoxicity in HEI-OC1 cells (Fig. 1B; 0.42 ± 0.11 vs. 0.71 ± 0.19; P<0.01). Therefore, this concentration (100 μg/ml PNS and 20 μM/l CP) was selected for use in subsequent experiments.

PNS reduces cisplatin-mediated apoptosis in HEI-OC1 cells. The ImageJ software version 1.8.0 (National Institutes of Health) was used to measure the rate of cell apoptosis. The induction of HEI-OC1 cell apoptosis was not evident in the control group. Following exposure to cisplatin, the number of HEI-OC1 cells that had undergone apoptosis was significantly increased compared with control or PNS group (Fig. 2A-D). This effect was significantly relieved following pretreatment of the cells with PNS (Fig. 2A-D). To further verify the anti-apoptotic effect of PNS, western blotting was used to assess the expression levels of apoptosis-associated proteins and the results indicated that HEI-OC1 cells treated with cisplatin exhibited increased expression levels of active caspase-3 and reduced Bcl-2 expression levels compared with the corresponding levels noted in the control group (Fig. 2E).

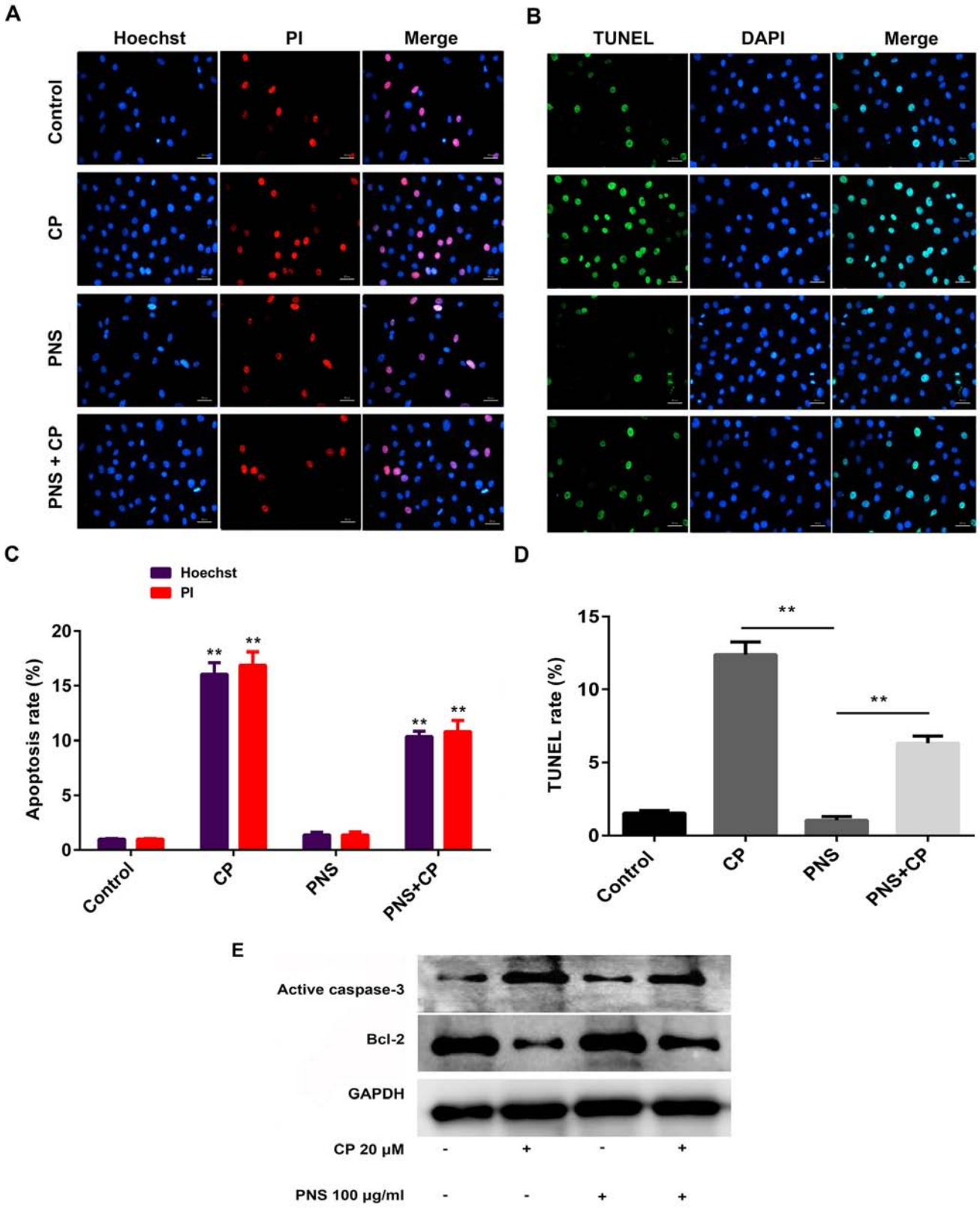

Figure 2. PNS attenuates CP-mediated apoptosis in HEI-OC1 cells. (A) Hoechst 33258 staining and PI staining of different groups. (B) TUNEL staining results in different groups. (C) Percentage of Hoechst 33258 positive staining and PI positive staining (n=3 per group). **P<0.01, CP vs. control; PNS + CP vs. CP. (D) Quantified results of TUNEL staining in different groups. **P<0.01, PNS + CP vs. CP; PNS vs. CP. (E) Western blot analysis of the protein levels of active caspase-3 and Bcl-2. GAPDH was used as an internal control for equal loading. All data are presented as the mean ± SEM. CP, cisplatin; PNS, *Panax notoginseng* Saponins; PI, propidium iodide.

PNS attenuates cisplatin-mediated ROS production and increases antioxidant enzyme expression. To further investigate the mechanism by which PNS attenuated apoptosis induction by cisplatin in HEI-OC1 cells, changes

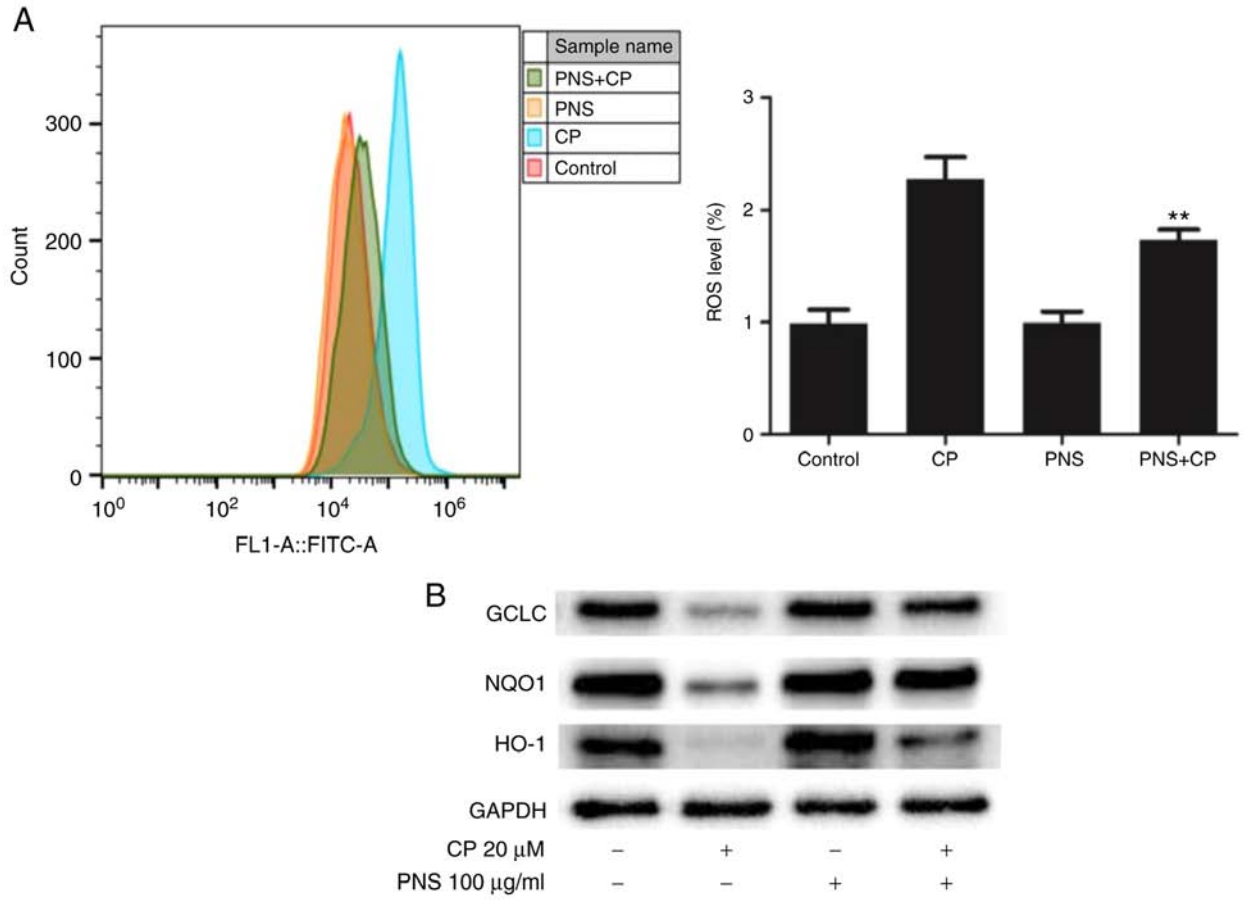

Figure 3. PNS attenuates cisplatin-mediated ROS production and increases antioxidant enzyme expression levels. (A) Flow cytometry results and statistical analysis of the data from the different groups. (B) Western blot analysis of the protein expression levels of GCLC, NQO1 and HO-1. GAPDH was used as an internal control for equal loading. All data are presented as the mean ± SEM. **P<0.01 vs. CP group. CP, cisplatin; PNS, *Panax notoginseng* Saponins; ROS, reactive oxygen species; GCLC, glutamate-cysteine ligase catalytic; NQO1, NAD(P)H quinone dehydrogenase 1; HO-1, heme oxygenase 1.

in the production of ROS and in the expression levels of antioxidant enzymes were examined. The ROS levels in the CP group was higher compared with the PNS + CP, PNS or control groups (Fig. 3). This suggested that PNS could significantly reduce the increase in ROS levels caused by cisplatin. Western blot analysis indicated that cisplatin could reduce the expression levels of GCLC, NQO1 and HO-1 (Fig. 3B). These results indicated that PNS could alleviate cisplatin-induced cell damage and apoptosis by restoring the balance between oxidative stress and the expression levels of antioxidant enzymes.

PNS increases Nrf2 expression by upregulating AKT phosphorylation in HEI-OC1 cells. A recent study has demonstrated that the AKT-Nrf2 axis regulates the expression of antioxidant enzymes (27). Initially, the effects of PNS pretreatment on Nrf2 expression were examined. Following cisplatin administration, Nrf2 expression in HEI-OC1 cells has no significant variation at 6 h, whereas it was markedly decreased at 12 and 24 h (Fig. 4A and C). However, HEI-OC1 cells pretreated with PNS expressed lower levels of Nrf2 at 6 and 12 h compared with the cisplatin group. In addition, the phosphorylation levels of AKT were examined after 6 h of PNS treatment of cells (Fig. 4B and D). PNS treatment increased AKT phosphorylation levels induced by cisplatin at 6 h. These results indicated that the protective effect of PNS on cisplatin-induced cytotoxicity may be achieved via the AKT/Nrf2 axis.

Protective effects of PNS on HEI-OC1 cells are blocked by AKT inhibition. To demonstrate that the inhibition of cisplatin-induced cytotoxicity by PNS was mediated via the AKT/Nrf2 axis, the AKT inhibitor LY294002 was used. Treatment of the cells with MK2002 blocked the protective effect of PNS on cisplatin-induced cytotoxicity (0.43±0.16 vs. 0.76±0.17, P<0.01; Fig. 5).

Discussion

Cisplatin is a complex of heavy metal platinum and was officially approved for clinical chemotherapy in the United States in 1972 (28,29). As one of the most widely used broad-spectrum antitumor drugs in clinical settings, cisplatin exerts potent antitumor activity and is widely used to treat malignant tumors of the head and neck (1,30,31). However, cisplatin causes ototoxicity that mainly manifests as deafness (2,3,32). This adverse effect limits its clinical application (2,3,32). Deafness is usually the most apparent cause of bilateral hearing impairment (33). As the dose of cisplatin increases, hearing loss can gradually develop from the low frequency

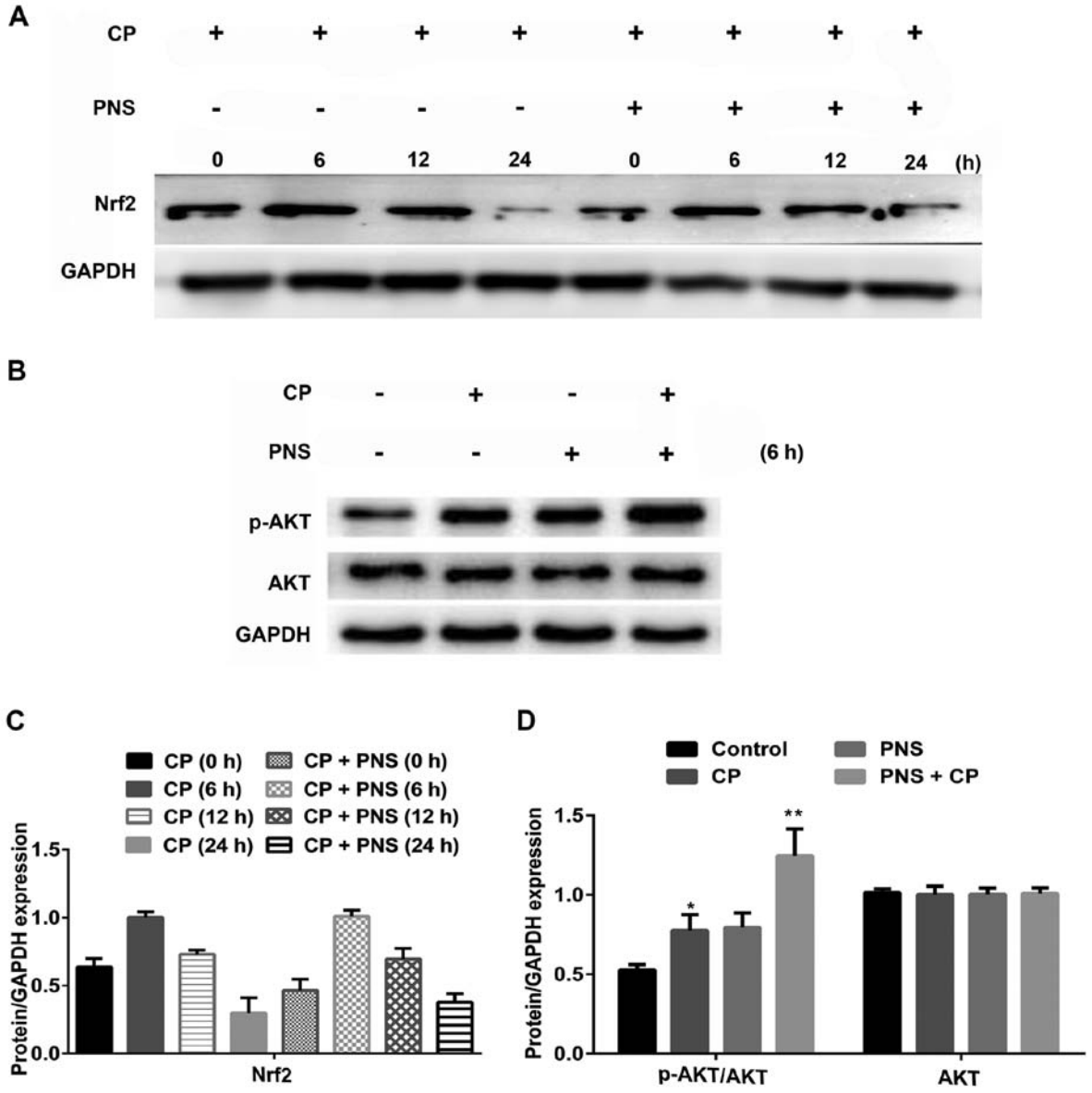

Figure 4. PNS increases Nrf2 expression by upregulating AKT phosphorylation in HEI-OC1 cells. (A) Western blot analysis of the protein expression levels of Nrf2 at different time points. (B) Western blot analysis of the protein levels of AKT and p-AKT at 6 h. (C) Quantified results of A are presented. (D) Quantified results of B are presented. GAPDH was used as an internal control for equal loading. *P<0.05 vs. control. **P<0.01, PNS + CP vs. CP; CP vs. control. PNS, *Panax notoginseng* Saponins; Nrf2, nuclear factor erythroid 2-related factor 2; p-, phosphorylated.

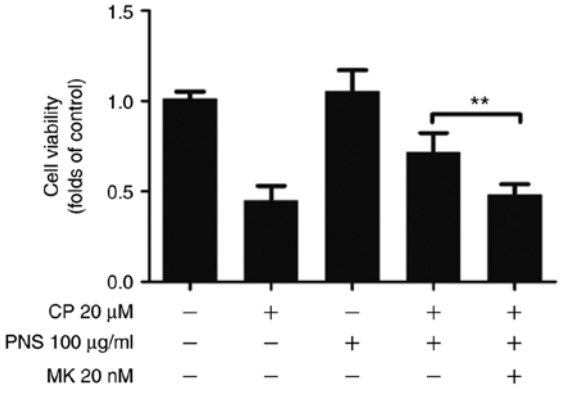

Figure 5. Protective effect of PNS on HEI-OC1 cells is blocked by AKT inhibitor treatment. HEI-OC1 cell viability was determined following treatment of the cells with 100 mg/ml PNS, 20 μM CP and 20 nM MK. All the data are presented as the mean ± SEM. **P<0.01. PNS, *Panax notoginseng* Saponins; CP, cisplatin; MK, MK2002.

region and affect the language frequency, eventually leading to permanent hearing loss (31). Accumulating evidence has demonstrated that cisplatin primarily destroys hearing by inducing auditory cell apoptosis (31-33). In the present study, the auditory HEI-OC1 cell line was used. Following culture with cisplatin, the viability of HEI-OC1 cells was reduced and the apoptotic rate was increased, which was consistent with observations from other studies (34,35).

PNS is an active ingredient extracted from *Panax notoginseng* (Sanqi). It contains >20 types of dammarane-type saponins and is prepared as a Xueshuantong capsule injection (36,37). PNS is commonly used to treat acute cerebral infarction and acute myocardial infarction (18,19). Previous studies have demonstrated that PNS can inhibit cisplatin-induced nephrotoxicity and neurotoxicity (20-23). However, whether PNS can inhibit ototoxicity induced by cisplatin has not yet been elucidated. The present study

demonstrated that PNS attenuated apoptosis induced by cisplatin in HEI-OC1 cells and increased their viability, which was consistent with the changes in the apoptosis-associated proteins Bcl-2 and caspase-3. The results indicated that PNS exhibited a therapeutic effect on ototoxicity induced by cisplatin.

At present, the underlying mechanisms of ototoxicity induced by cisplatin have not been fully demonstrated, whereas the excessive induction of oxidative stress and auditory cell apoptosis caused by increased free radical production has been widely accepted (3,10,38). Previous studies have reported that cisplatin can produce an excess of free radicals and deplete the activity of antioxidant enzyme systems in the cochlea, resulting in DNA, lipid and protein damage, as well as hair cell apoptosis, via the mitochondrial signaling pathway (38-40). Therefore, the restoration of the balance between oxidative stress and the expression of antioxidant enzymes is considered an important way to treat cisplatin-induced ototoxicity (4). Previous studies have demonstrated that PNS is dependent on potent free radical scavenging and antioxidant action in order to exert its therapeutic effects on cardiovascular and cerebrovascular conditions (36,41,42). In the present study, PNS administration could inhibit ROS production in HEI-OC1 cells exposed to cisplatin. Further experiments revealed that the protein expression levels of the antioxidant enzymes, including HO-1, NQO1 and GCLC, were markedly increased following PNS pretreatment, which indicated that remission of HEI-OC1 cell apoptosis may be associated with PNS-induced restoration of ROS balance.

Nrf2 is a transcription factor that exerts its function by regulating the redox homeostatic gene network (43). A previous study demonstrated that the activated Nrf2 protein binds to the antioxidant response element and increases the transcription of various cytoprotective genes that encode antioxidant proteins, including NQO1, HO-1 and GCLC (27). The increase in the expression levels of antioxidant enzymes, including GCLC, NQO1 and HO-1, could counteract the induction of oxidative stress (11-14). These enzymes reduce the intracellular ROS levels and, therefore, protect cells from oxidative damage (44,45). Additionally, the Nrf2 protein can upregulate the expression levels of Bcl-2 in order to prevent cell apoptosis (31). In the present study, PNS increased the levels of AKT phosphorylation and Nrf2 expression. These effects were reversed by treatment of the cells with an AKT inhibitor. These results suggested that PNS protected against ototoxicity induced by cisplatin via the activation of the AKT/Nrf2/ signaling-mediated redox pathway.

However, *in vivo* experiments should be conducted in the future in order to further discover the underlying effects of PNS on regulation of cisplatin-induced ototoxicity in auditory cells, which is considered to be a limitation of the present study.

In conclusion, the present study indicated the potential mechanism of the anti-cytotoxic and anti-apoptotic effects of PNS against cisplatin-induced ototoxicity. This mechanism was mediated by activation of the AKT/Nrf2 signaling pathway. The results indicated that PNS may function as a promising candidate in the prevention and treatment of cisplatin-induced ototoxicity.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BF, ZBL, LSX, LYL, WYZ, JL, YHD and WDS made substantial contributions to conception, design and acquisition of data. BF, LYL, WYZ and JL contributed to manuscript drafting, analysis and interpretation of data. YHD and WDS were involved in manuscript revising, general supervision. All authors read and approved the final manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy of integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School and in accordance with the Declaration of Helsinki. Written consent was obtained from each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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