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# Allicin protects auditory hair cells and spiral ganglion neurons from cisplatin - Induced apoptosis

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ABSTRACT

Cisplatin is a broad-spectrum anticancer drug that is commonly used in the clinic. Ototoxicity is one of the major side effects of this drug, which caused irreversible sensorineural hearing loss. Allicin, the main biologically active compound derived from garlic, has been shown to exert various anti-apoptotic and anti-oxidative activities in vitro and in vivo studies. We took advantage of C57 mice intraperitoneally injected with cisplatin alone or with cisplatin and allicin combined, to investigate whether allicin plays a protective role in vivo against cisplatin ototoxicity. The result showed that C57 mice in cisplatin group exhibited increased shift in auditory brainstem response, whereas the auditory fuction of mice in allicin + cisplatin group was protected in most frequencies, which was accordance with observed damages of outer hair cells (OHCs) and spiral ganglion neurons (SGNs) in the cochlea. Allicin markedly protected SGN mitochondria from damage and releasing cytochrome c, and significantly reduced pro-apoptosis factor expressions activated by cisplatin, including Bax, cleaved-caspase-9, cleaved-caspase-3and p53. Furthermore, allicin reduced the level of Malondialdehyde (MDA), but increased the level of superoxide dismutase (SOD). All data suggested that allicin could prevent hearing loss induced by cisplatin effectively, of which allicin protected SGNs from apoptosis via mitochondrial pathway while protected OHCs and supporting cells (SCs) from apoptosis through p53 pathway.



## 1. Introduction

Cisplatin is an effective and broad-spectrum anticancer drug that is commonly used in the treatment of various human cancers, including carcinoma of the head and neck region (Kalcioglu et al., 2005; Goncalves et al., 2013). However, ototoxicity induced by cisplatin leads to irreversible high-frequency sensorineural hearing loss (Hartmann and Lipp, 2003). Studies reported that cisplatin mainly targets three areas in the cochlea: the sensory hair cells in the organ of Corti (Anniko and Sobin, 1986), the lateral wall tissues (Meech et al., 1998) and the spiral ganglion neurons in the modiolus (van Ruijven et al., 2005). Although the cytotoxic mechanism of cisplatin is not fully clear, a large number of studies have proved that the apoptosis is closely involved in DNA damage, oxidative stress and inflammatory factors (Rybak et al., 2007; Jamesdaniel et al., 2008; Park et al., 2009; Schmitt et al., 2009). Therefore, antioxidants and anti-apoptotic agents, as two major groups of molecules, are commonly investigated to evaluate the ototoxicity induced by cisplatin in the cochlea (Duan et al., 2002).

Many studies showed that allicin, a major ingredient of fresh garlic extract, played roles in antimicrobial (Cutler and Wilson, 2004; Canizares et al., 2004), antitumor (Park et al., 2005; Patya et al., 2004), antioxidant (Borek, 2001; Liu et al., 2015a), anti-inflammatory (Lang et al., 2004; Hodge et al., 2002) and anti-apoptosis activities (Zhang et al., 2008). Existing research data have shown that allicin induced the antioxidant and protected cells against oxidative stress, by reducing cytotoxic substances and scavenging free radical (Chan et al., 2013). Recently, it is

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demonstrated that allicin possess neuroprotective activity on traumatic or ischemic neuronal injury which is regulated by oxidative stress and apoptosis pathways (Liu et al., 2015a; Chen et al., 2014a; Zhou et al., 2014). A previous study also confirmed that allicin attenuated spinal cord ischemia–reperfusion injury through improving the function of mitochondrion (Zhu et al., 2012). However, there are no report to date of anti-apoptotic and anti-oxidative effects of allicin on ototoxicity induced by cisplatin. The aim of the present study therefore is to determine whether allicin would be effective in alleviating hearing loss induced by cisplatin.

## 2. Materials and methods

### 2.1. Reagents

Cisplatin was purchased from Jiangsu Haosen pharmaceutical Co. Ltd (Jiangsu, China) and dissolved in 0.9%physiological saline. Allicin was purchased from Xuzhou Lai'en Pharmaceutical Co. Ltd. (Shandong, China) and dissolved in 0.9%physiological saline. Antibodies to Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9 were acquired from Cell Signaling Technology (Beverly, MA, USA). Antibody to Tuj 1 was acquired from Neuromics (Edina, USA). Antibody to cytochrome c, actin and P53 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Click-iT Plus TUNEL assay kits was purchased from Life Technologies (Invitrogen, USA). Superoxide dismutase (SOD) and malonaldehyde (MDA) assay kits were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA protein assay kit was a product from Beyotime (Shanghai, China).

### 2.2. Experimental animals

All experiments were performed according to protocols approved by the Animal Care Committee of Shandong University on the care and use of Laboratory Animal for Research Purposes. C57 mice were purchased from Animal Center of Shandong University (Jinan, China). All animals were bred and housed in standard box cages in a climate-controlled room with an ambient temperature of $23 \pm 2$ °C and a 12/12 h light/dark cycle. Animals were fed standard laboratory chow, given water freely and were assigned randomly to control or experimental groups. Experiments were performed on age- and sex-matched 7- to 8-week-old mice weighing 17–23 g. The mice were divided into three groups ($n = 30$ each; group 1, 0.9% physiological saline-injected controls; group 2, cisplatin-injected; group 3, cisplatin + allicin-injected). Mice in groups 1, 2 and 3 received intraperitoneal (i.p.) injections of 0.9% physiological saline (0.6 ml/100 g), cisplatin (3 mg/kg) or allicin (18.2 mg/kg), respectively. Group 1 mice were administered with 0.9% physiological saline (0.6 ml/100 g i.p.) for seven consecutive days. Group 2 mice were administered with cisplatin (3 mg/kg i.p.) for seven consecutive days. To evaluate the effects of allicin on cisplatin-induced ototoxicity, groups 3 mice were given 18.2 mg/kg allicin i.p. one day ahead and at 2 h before the daily injection of cisplatin.

### 2.3. Measurement of auditory brain stem response

The auditory brain stem response (ABR) was measured before and after seven day injection. TDT system hardware and software (Tucker-Davis Technologies, Alachua, FL,USA) were used to record ABRs, with 1024 stimulus repetitions per record. Mice were anesthetized with a chloral hydrate (400 mg/kg) and kept warm during ABR recordings. The record electrode was inserted into subcutaneous tissue at the vertex, reference and ground electrodes were placed subcutaneously at ipsilateral mastoid and back, respectively. Tone bursts of 4 ms duration with a rise–fall time of 1 ms at frequencies of 4,8, 12,16, 24 and 32 kHz were presented to the left ear through a metal loudspeaker in the external auditory meatus. The sound intensity was varied at 10 dB intervals near threshold. Threshold Judgment of three groups was made by the same person.

### 2.4. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) assay

TUNEL were performed according to the manufacturer's instruction of Click-iT Plus TUNEL assay kits. At last, each section were stained with DIPA(1:1000) solution for 15 min at 37 °C and protected from light. After washing with PBS, samples were examined using a laser scanning confocal microscope (Leica, Germany).

### 2.5. Survival outer hair cell (OHC) and spiral ganglion neuron (SGN) counting

#### 2.5.1. OHC counting

Seven days after drug administration, C57 mice were anesthetized with lethal doses of chloral hydrate and the cochleae were harvested. After fixation for 2 h and decalcification with 0.5 M EDTA for 6 h, cochleae were dissected under a microscope by removing the lateral wall of the cochlea, spiral ligament and modiolus. The remaining tissue was cochlear sensory epithelium. The epithelia were divided into three segments (apex, middle, and base) and stuck to a small glass slide. The OHCs were evaluated in three groups ($n = 4$ per group). The residual OHCs were counted in a 1 mm long strip at the region from base to apex.

#### 2.5.2. SGN counting

Seven days after drug administration, C57 mice were anesthetized with lethal doses of chloral hydrate and the cochleae were harvested and perfused with 4% paraformalde-hyde in PBS. After decalcification and dehydration, The tissues were embedded by OCT in dry ice for frozen section. The spiral ganglion cells were evaluated among three groups ($n = 5$ per group). The spiral ganglion cell countings were performed within the unit area in three sections from each cochlear in apical, middle and basal turns.

### 2.6. Transmission electron microscopy (TEM) assessment of outer hair cell and spiral ganglion cell

Briefly, animals were decapitated under deep anesthesia, and the cochleae tissue was removed, washed fast with PBS, immediately placed in 3% glutaraldehyde fixative solution (pH 7.4), the sample block trimming 1 mm × 1 mm × 3 mm, according to the conventional TEM sample preparation method followed by rinsing, 1% osmic acid (OsO4) fixed, rinsing, dehydration, soaked, epon812 embedded; semi- and ultra-thin radial sections were cut from the basal and middle turns with lead citrate and uranyl acetate electron staining. Finally, the sections were observed using a transmission electron microscope (JEOL-1200EX) in JiNan WeiYa Bio-Technology Co, Ltd. (Jinan, China).

### 2.7. Western blotting

Seven days after drug administration, the proteins from mice cochleae were extracted and expressions of cleaved-caspase-9, cleaved-caspase-3, Bax and Bcl-2 genes were examined by Western blot as described (Liu et al., 2011). Briefly, total protein was extracted from cochleae using radio-immune precipitation buffer protein lysis buffer. The protein content of the samples was measured using the BCA protein assay kit. 30 μg of each protein sample was separated by 12% SDS-PAGE gels. The primary antibodies were (anti-Bcl-2, 1:1000; anti-Bax, 1:1000; anti-cytochrome

c, 1:500; anti-cleaved-caspase-9, 1:1000; anti-cleaved-caspase-3, 1:1000; anti-Actin, 1:10000). The relative optical density ratio of Bcl-2, Bax, cytochrome c, cleaved-caspase-9 and cleaved-caspase-3 to Actin were calculated with the Image J software.

### 2.8. Immunofluorescence staining

Each sample was performed as described previously (Garcia-Berrocal et al., 2007). The primary antibodies were cleaved-caspase-3 (1:400), cleaved-caspase-9 (1:400), cytochrome c (1: 50), P53 (1: 50), Tuj-1 (1: 1000, Neuromics) and Sox-2 (1:50, Santa Cruz, CA). The next day, tissues were incubated with FITC-conjugated or TRITC-conjugated (1: 1000, Invitrogen) donkey anti-goat, anti-mouse, or anti-rabbit secondary antibodies (1:1000, Life Technologies, Carlsbad, CA) along with diamidino-phenyl-indole (DAPI 1:1000, Sigma-Aldrich) or phalloidin (1: 1000, Sigma-Aldrich) staining at room temperature in darkness for 1 h. Specimens were mounted on slides imaged on a Leica confocal microscope (Leica, Germany).

### 2.9. Detection of the level of MDA and antioxidant enzymes activities by spectrophotometer

The whole mice cochlear extracts were obtained to detecting the levels of MDA and superoxide dismutase (SOD) activity. The level of MDA and SOD activity in all groups were measured by commercial assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). All experiments were repeated three times.

### 2.10. Statistical analysis

The experiments shown are a summary of the data from at least three experiments and all statistical analysis was performed by GraphPad Prism software. Results were expressed as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newmane Keuls multiple-comparison post hoc test. $P$ value < 0.05 was considered statistically significant.

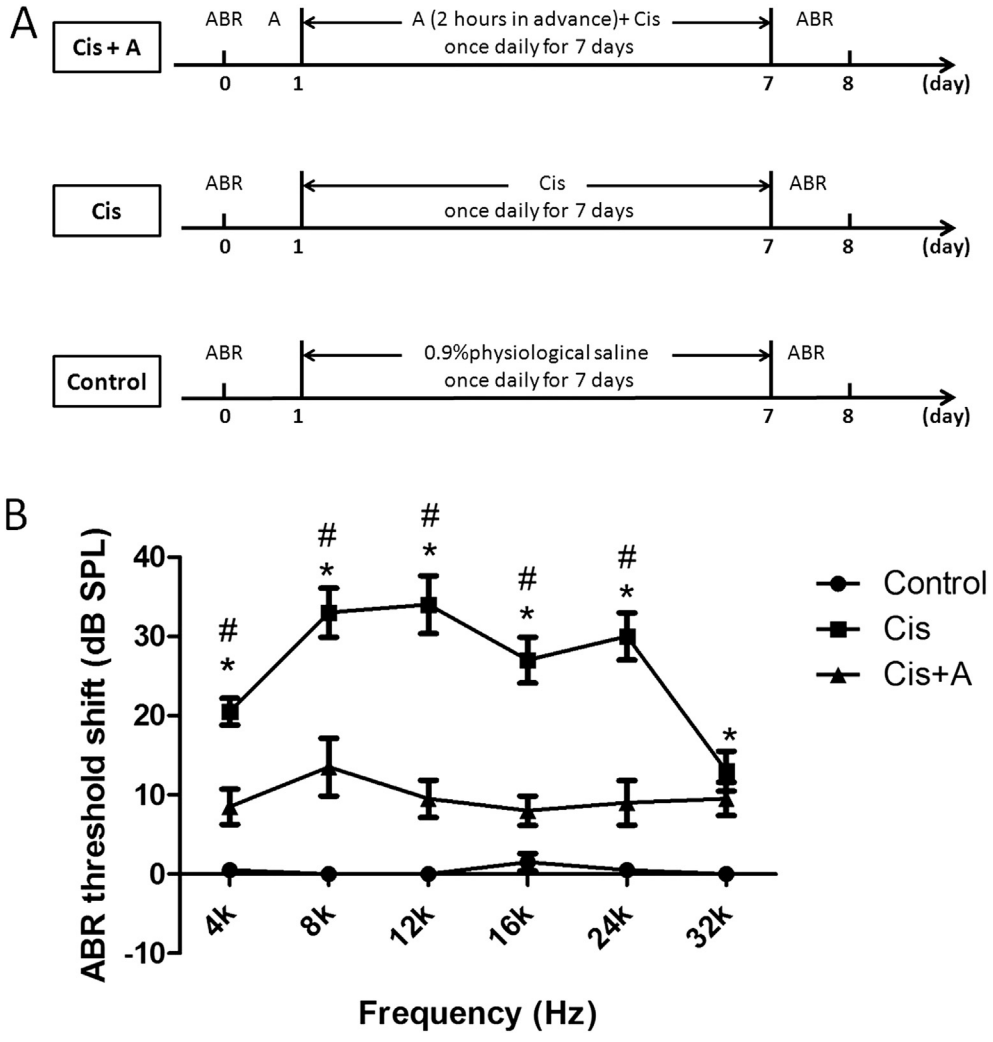


**Fig. 1.** The effects of allicin on ABR thresholds in cisplatin-treated C57 mice. (A) C57 mice were divided into three groups (each group, n = 20) and given intraperitoneal injections of drugs as illustrated in A. (B) The ABR threshold shifts in cisplatin group were increased at 4 k, 8 k, 12 k, 16 k, 24 k and 32 kHz respectively compared to the control group. In pre-treated with allicin group, the ABR threshold shifts were obviously decreased in 4 k, 8 k, 12 k, 16 k, 24 k Hz except 32 kHz compared to the cisplatin group. Mean ± SEM; *P < 0.05 vs. control group; #P < 0.05 vs. Cis + A group; Control, 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin.

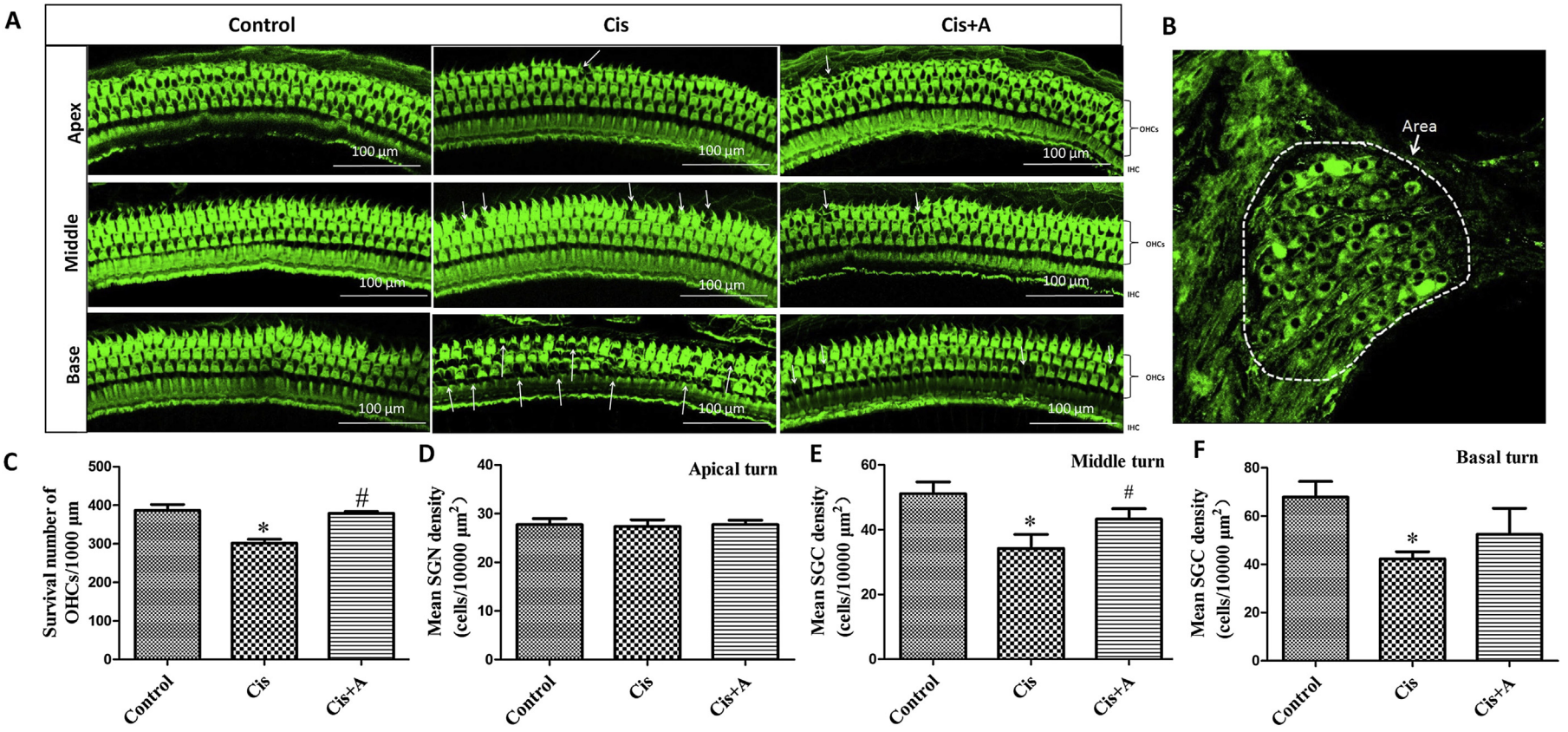


**Fig. 2.** The effects of allicin on the survival number of outer hair cells (OHCs) and spiral ganglion neurons (SGNs) in cisplatin treatment mice. (A) Representative pictures of hair cells labeled with Alexa Fluor 488 phalloidin (green) in the apical, middle and basal turns of cochleae of C57 mice after drug treatment. The missing hair cells were marked with arrows. (B) The illustration of SGN counting (Tuj-1, green). The SGN density means a ratio of SGN numbers to area. The dotted line indicates area of SGNs. (C) Quantitative analysis of the survival OHCs (each group, n = 4). The survival number of OHCs in cisplatin group was 302/1000 μm, which was decreased significantly compared with the control group 387/1000 μm (*P < 0.05). In cisplatin + allicin group, the survival number of OHCs was increased to 379/1000 μm, which was significantly different compared with cisplatin group (#P < 0.05). (D) Quantitative analysis of the survival number of SGNs (each group, n = 5). There was no significant difference in the apical turn among three groups. (E)In the middle turn of the cochlea, the mean density of SGNs in cisplatin group was only 34/10000 $\mu m^2$ while it was 51/10000 $\mu m^2$ in control group. The difference has statistical significance (*P < 0.05). In cisplatin + allicin group, it was 43/10000 $\mu m^2$, which was increased significantly compared with cisplatin group (#P < 0.05). (F) In the basal turn of the cochlea, the mean density of SGNs in three groups were 67/10000 $\mu m^2$, 42/10000 $\mu m^2$ and 52/10000 $\mu m^2$ respectively. The comparison between cisplatin and control group was significantly different (*P < 0.05), nevertheless, comparison between cisplatin and cisplatin + allicin group was no statistical significance (#P > 0.05). Data are presented as mean ± SEM; *P < 0.05 vs. Control group; #P < 0.05 vs. Cis group; Control, 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin.

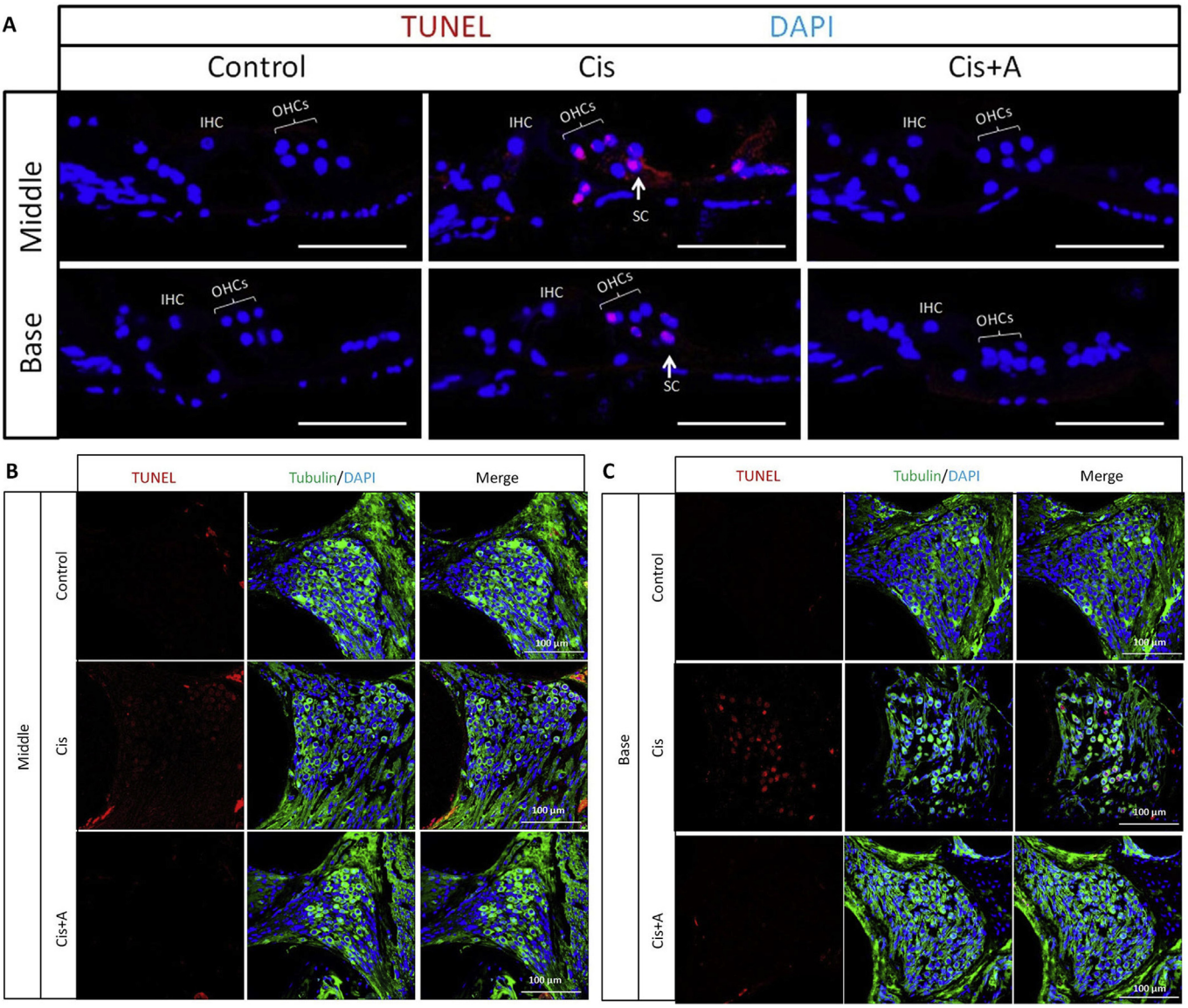


**Fig. 3.** The protective effect of allicin against cisplatin in cochleae by TUNEL staining for apoptosis. Seven-micrometer cochlear sections from each experimental group were stained using TUNEL and then observed using confocal (each group, n = 3). Positive stainings of TUNEL could be found in OHCs, SCs (arrows in A) and SGNs (B and C) in middle and basal turns. However, few positive staining of TUNEL could be found in cisplatin + allicin group in those structures. No positive staining of TUNEL in the control group. Control, 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin; OHCs, outer hair cells; IHC, inner hair cell; SCs, supporting cells; Scale bars, 50 μm in A; 100 μm in B and C.

## 3. Results

### 3.1. Auditory brainstem response (ABR) recordings

7-8-week adult C57 mice were chosed to examine the effect of cisplatin on hearing loss. Before the drug treatment, ABR test were performed to make sure that all the C57 mice had normal auditory function. The result showed that after 7 days with drug treatment, the ABR threshold shifts in cisplatin administration group were increased compared to the control group (Fig. 1, $^{*}P < 0.05$), suggesting that cisplatin impaired mouse auditory function in all the frequencies we examined. However, in pre-treated with allicin group, the ABR threshold shifts were obviously decreased in most frequencies except 32 kHz, indicating the protection of allicin against ototoxicity induced by cisplatin (Fig. 1).

### 3.2. The survival number of outer hair cells(OHCs) and spiral ganglion neurons(SGNs)

We first analyzed the survival number of outer hair cells (OHCs) in three groups (Fig. 2A and C). The survival number of OHCs in cisplatin group was decreased significantly compared with the control group. However, in cisplatin + allicin group, the survival number of OHCs was increased, which was significantly different compared with cisplatin group.

Then we analyzed the survival spiral ganglion neurons (SGNs) of the apical, middle and basal turns in the three groups. There was no significant difference in the apical turn among three groups (Fig. 2D). In the middle turn of the cochlea (Fig. 2E), the mean density of SGNs in cisplatin group was lower than that in control group. The difference has statistical significance ($P < 0.05$). In cisplatin + allicin group, the density was increased significantly

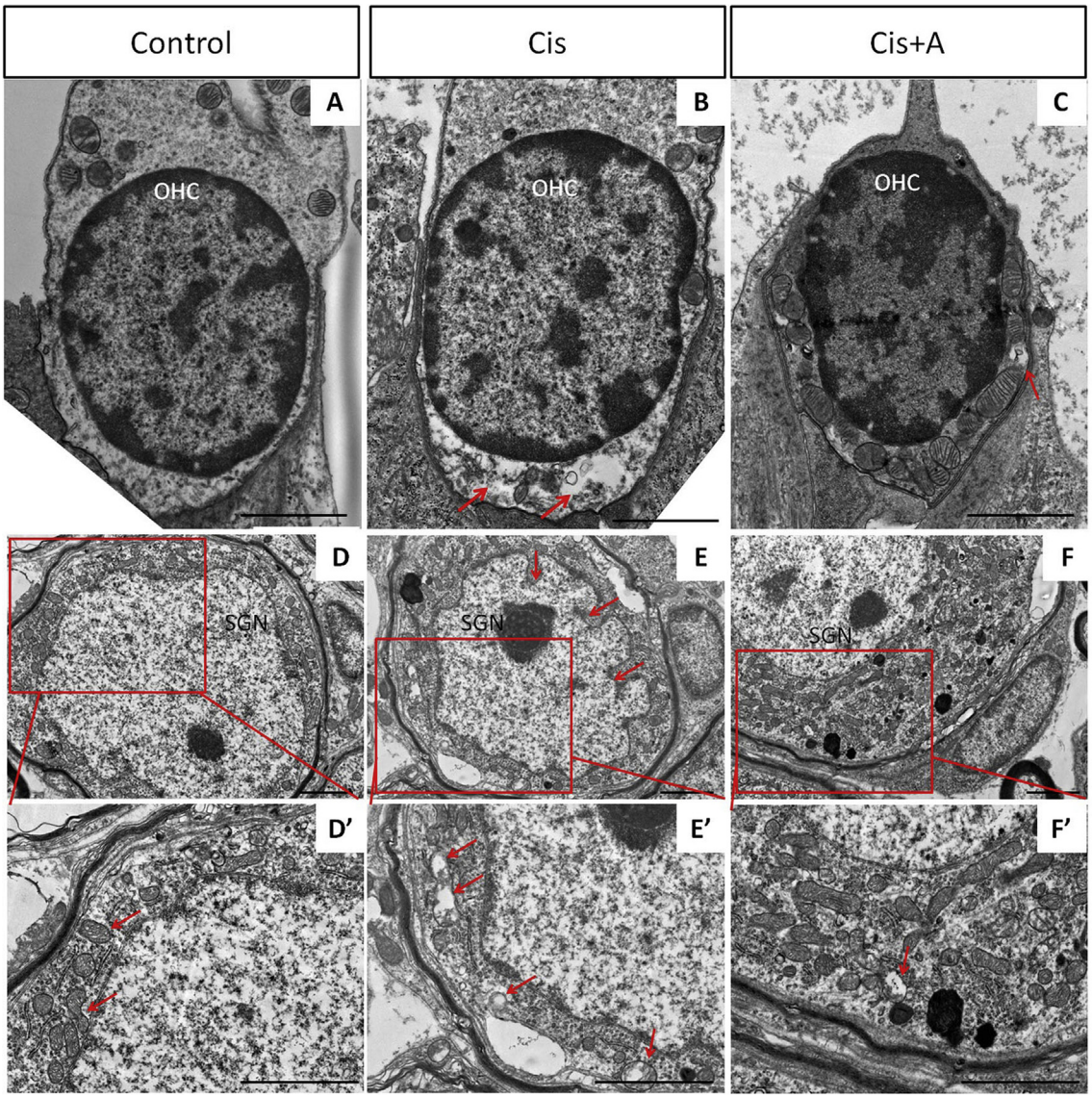


**Fig. 4.** Protective effect of allicin on OHCs and SGNs observed by transmission electron microscopy (TEM). OHCs (A, B and C) and SGNs (D, E and F) in three groups. Panel D′, E′ and F′ were magnified areas as illustrated by red rectangles in D, E and F respectively. In control group, the density in the cytoplasm of OHC(A) and SGN(D) was uniform. The mitochondria were normal and mitochondrial cristae were clear and arranged normally (red arrows in D′). However, in cisplatin group, mitochondria in SGNs were obviously damaged which swelled and showed vacuolate degeneration (red arrows in E′), the nuclear membrane invaginated (red arrows in E). The large area of low electron density region in the OHC cytoplasm could be seen (red arrows in B), indicating swelling of hair cell body. In allicin + cisplatin group, damage of mitochondria caused by cisplatin was reduced and mild (red arrows in F′). A low electron density region was occasionally seen in the cytoplasm of OHC (red arrow in C). Mitochondria in OHCs of the basal turn appear normal among three groups. Each group, n = 2; Control, 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin; OHCs, out hair cells; SGNs, spiral ganglion neurons; Scale bars, 2 μm.

compared with cisplatin group. It presented a similar trend in basal turn (Fig. 2F). The comparison between cisplatin and control group was significantly different, nevertheless, comparison between cisplatin and cisplatin + allicin group was not statistically significant.

### 3.3. Detection of nuclear DNA fragmentation

In cisplatin group, positive stainings of TUNEL could be found in OHCs, supporting cells (SCs), SGNs in middle and basal turns (Fig. 3). However, few positive staining of TUNEL could be found in the middle or basal OHCs, SCs or SGNs of the cisplatin + allicin group. No positive staining of TUNEL in the control groups.

### 3.4. Transmission electron microscopy (TEM) assessment of OHCs and SGNs

In control group, the morphology of mitochondria in SGNs was normal, and the mitochondrial cristae were clear and arranged normally. Whereas, in cisplatin group, many mitochondria in SGNs were obviously damaged with swelling, vacuolization, and the invagination of the nuclear membrane could be found. In cisplatin + allicin group, swelling of mitochondria was only occasionally seen, abnormal nuclear membrane could not be found (Fig. 4).

Mitochondria of OHCs did not change obviously in all groups. However, there was a low electron density region in cytoplasmic basal part of OHCs in cisplatin group (Fig. 4), implying the swelling of the cell body.

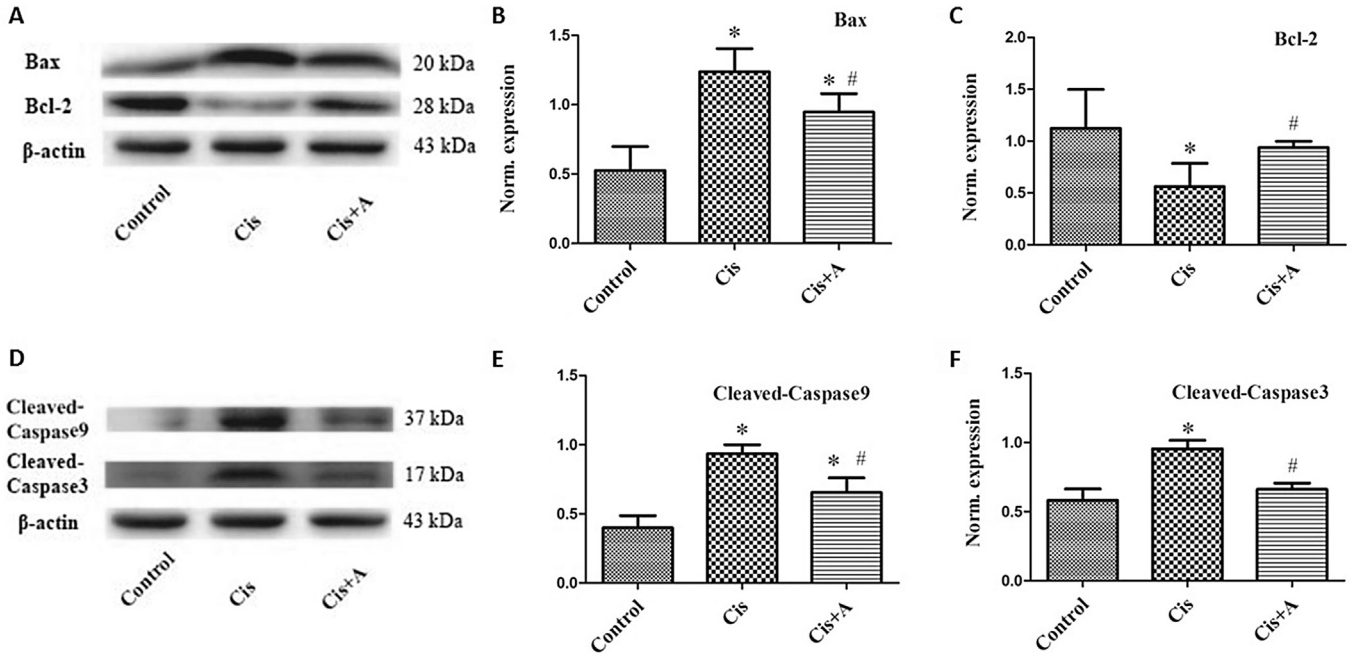


**Fig. 5.** Effects of allicin on protein expressions of Bax, Bcl-2, cleaved-caspase-9, and cleaved-caspase-3 in the cochlear tissues. Bax, cleaved-caspase-9 and cleaved-caspase-3 in cisplatin group were significantly higher than that in control group (A, B, D, E, F), while the expression of Bcl-2 in cisplatin group was significantly lower than that in control group (A, C). Notably, allicin significantly reduced the expressions of Bax, cleaved-caspase-9 and cleaved-caspase-3, meanwhile markedly increased the expression of Bcl-2 induced by cisplatin. *,#$P < 0.05$ by one-way ANOVA compared with the control (*) and cisplatin group (#). Control: 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin (each group, $n = 3$).

### 3.5. Western blot in the cochlea

Western Blot results showed that the protein expressions of pro-apoptosis factor Bax, cleaved-caspase-9, cleaved-caspase-3 in cisplatin group were significantly higher than that in control group (Fig. 5A,B,D,E,F), while the expression of anti-apoptotic factor Bcl-2 in cisplatin group was significantly lower than that in control group (Fig. 5A,C). Notably, allicin significantly reduced the expressions of Bax, cleaved-caspase-9 and cleaved-caspase-3, meanwhile markedly increased the expression of Bcl-2 induced by cisplatin, suggesting that allicin was through apoptosis inhibiting to protect against cisplatin ototoxicity.

### 3.6. Immunofluorescence in the cochlea

First, cytochrome c expression was examined. Immunofluorescence showed that in physiological saline group, cytochrome c was uniformly distributed in SGNs. In cisplatin group, the staining for cytochrome c displayed uneven distribution, and increased expression in the cytoplasm compared with control group. In cisplatin + allicin group, the staining for cytochrome c presented more uniform and decreased expression than that in cisplatin group (Fig. 6A).

Second, the expressions of cleaved-caspase-9 and cleaved-caspase-3were examined. In physiological saline group, there was no staining for cleaved-caspase-9 and cleaved-caspase-3 in the cytoplasm of SGNs. In cisplatin group, the staining for cleaved-caspase-9 (Fig. 6B) and cleaved-caspase-3 (Fig. 6C)were significantly enhanced. However, in cisplatin + allicin group, there were a few staining for cleaved-caspase-9 and cleaved-caspase-3 in SGNs. The staining for cleaved-caspase-3 could not be detectable in OHCs among all groups (date not show).

Finally, p53 expression was examined in supporting cells (SCs) and OHCs in the same region but different planes of Z-axis. Myosin7a and Sox2 were used as makers to label OHCs and SCs respectively. In physiological saline group, p53 expression could be detected neither in OHCs nor in SCs. In cisplatin group, the staining of p53 was positive both in nucleus of OHCs and SCs in basal and middle turns (Fig. 7A and B). In cisplatin + allicin group, only few p53-positive OHCs could be found in the base (Fig. 7A), but almost all the SCs were p53-positive in the base (Fig. 7A), indicating that SCs were more susceptible to cisplatin, and the protective effects of allicin on the OHCs were obvious. No staining is detectable in the apical turn either in OHCs plane or in SCs plane (Fig. 7C).

### 3.7. Detection the level of malondialdehyde (MDA) and superoxide dismutase (SOD) in the cochlea

Compared with the physiological saline group, the MDA level in cisplatin group was significantly increased, but the level of MDA in the cisplatin + allicin group decreased significantly compared with cisplatin group (Fig. 8A). On the contrary, compared with the control group, the SOD level in cisplatin group reduced significantly, but the SOD level in cisplatin + allicin group increased obviously compared with cisplatin group (Fig. 8B).

## 4. Discussion

### 4.1. Allicin's potential for protection against ototoxicity induced by cisplatin

Due to the side effects of cisplatin that limits its clinical application, many researchers try to reduce its ototoxicity. Not a few studies have shown that various antioxidants (Rybak et al., 2007), such as resveratrol (Yumusakhuylu et al., 2012),D-methionine (Korver et al., 2002), N-acetyl cysteine (Thomas Dickey et al., 2004), lipoic acid (Rybak et al., 1999a, b), amifostine (Fouladi et al., 2008), Bucillamine (Kim et al., 2015) and Sodium thiosulphate (Viallet

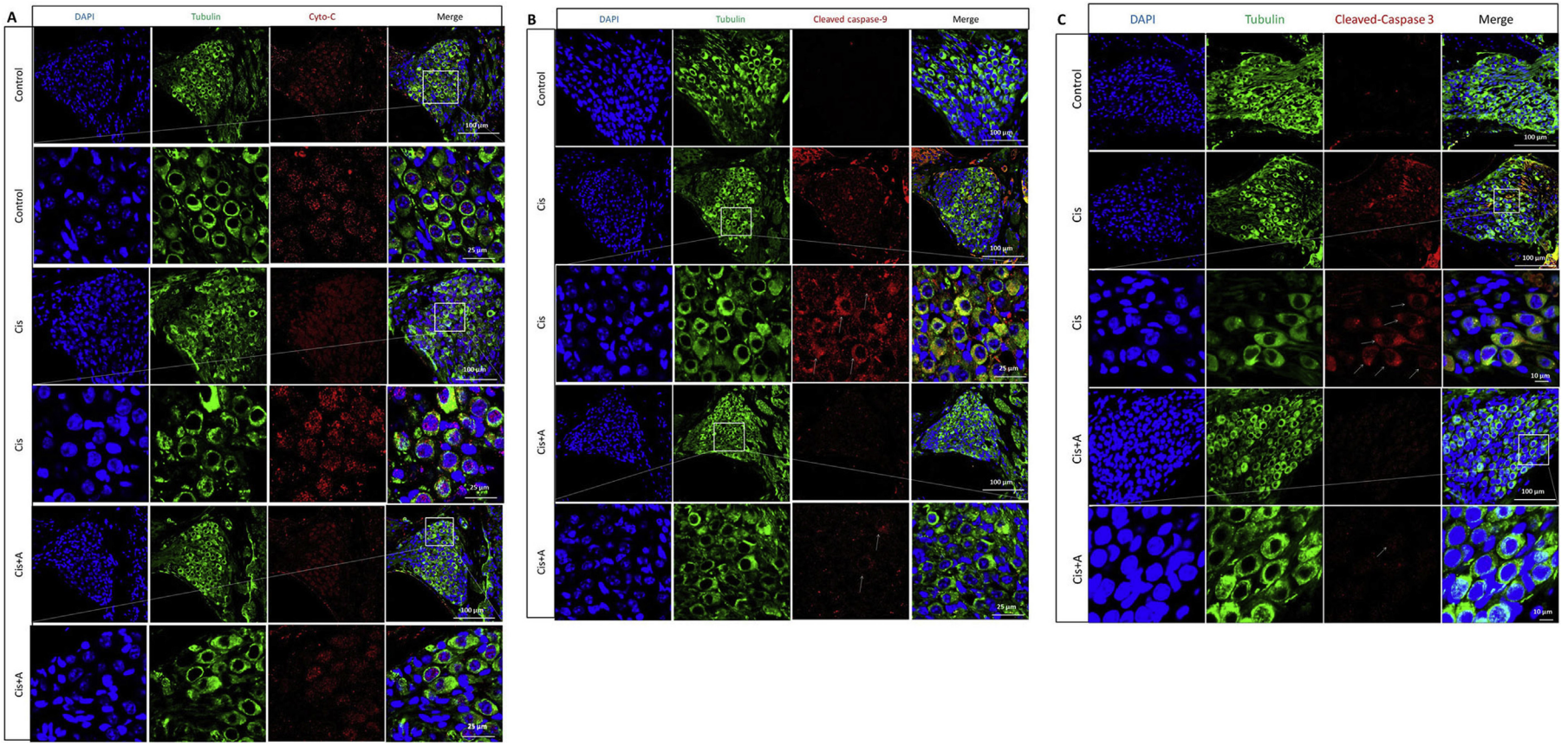


**Fig. 6.** Effect of allicin on protein expressions of cytochrome c, cleaved-caspase-9, cleaved-caspase-3 in spiral ganglion neurons (SGNs) of the basal turn. In the control group, cytochrome c was distributed uniformly as puncta. The staining of cleaved-caspase-9 and cleaved-caspase-3 were negative. On the contrary, in the cisplatin group, cytochrome c was no longer uniform in the cytoplasm. Cleaved-caspase-9 (arrows in B) and cleaved-caspase-3 (arrows in C) were expressed strongly in the cytoplasm of many SGNs. Notably, allicin significantly weakened the staining of cleaved-caspase-9 and cleaved-caspase-3 in the allicin treated group (arrows in B and C). At the same time cytochrome c appeared normal. Control, 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin (each group, n = 3).

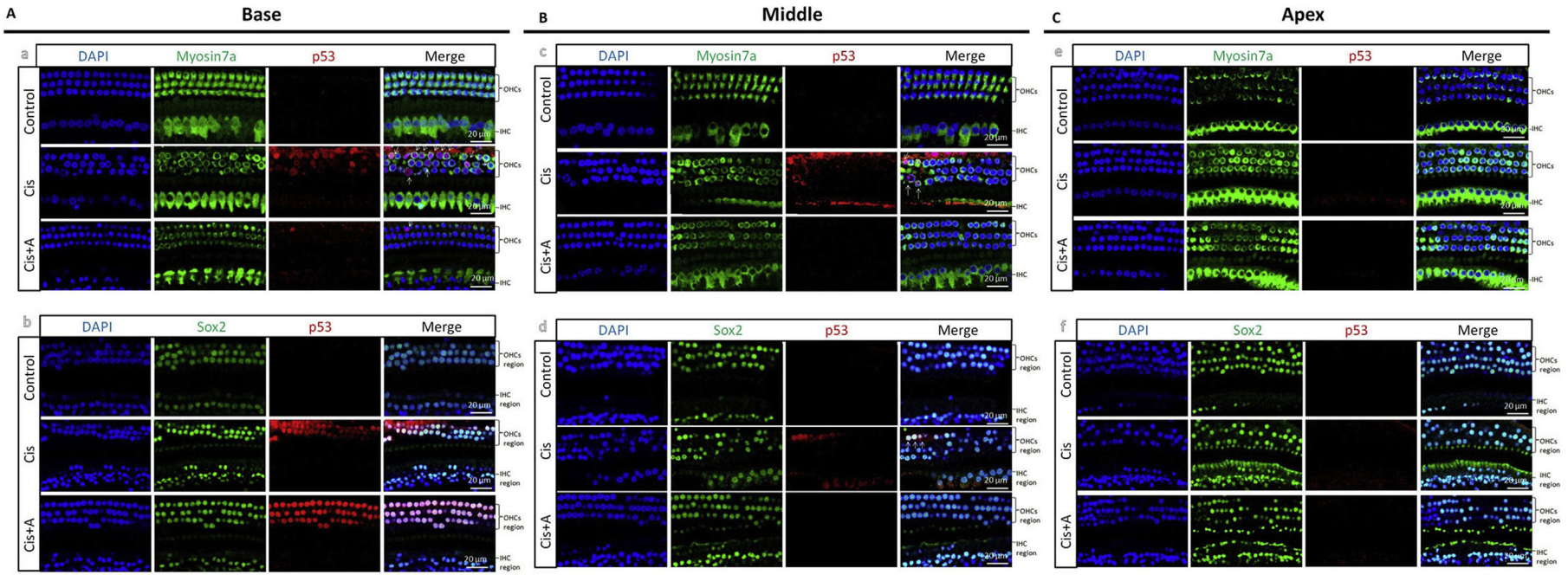


**Fig. 7.** The protective effect against cisplatin of allicin treatment in hair cells and supporting cells by p53 staining. Basal, middle and apical turn among three groups were observed respectively (A, B and C). The layers of hair cells (a, c, e) and supporting cells (b, d, f) in the Z-stack were marked by myosin7a and sox2 separately. In the control group, the three rows of outer hair cells (OHCs), one row of inner hair cells (IHCs) and supporting cells (SCs) can be clearly seen. No p53 staining was found. In cisplatin group, p53 staining could be found in the OHCs (arrows in a, c) and SCs (arrows in b, d) of the middle and basal turn of the cochlear. No p53 staining is detectable in the apical turn. But in allicin group, p53 staining only be found in the basal OHCs (arrows in a) and SCs (arrows in b),Control, 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin (each group, n = 3); Scale bars, 20 μm.

et al., 2006; Leitao and Blakley, 2003) could protect against cisplatin-mediated hearing loss. Although it is reported that allicin is an ideal antioxidant, there is no report whether allicin exerts protection against cisplatin ototoxicity.

In this study, although not obvious at 32 KHz frequency, it was found that pre-intraperitoneal injection of allicin could partly protect mouse auditory function. As we all know, normal auditory function needs intact auditory pathway, including crosstalk from hair cells to SGNs and to auditory nerve, also including the nutritional and structural supplies of SCs. A former study has documented that cisplatin could cause loss of OHCs in the cochlea, starting in the base and progressing to the apex of the cochle (Garcia-Berrocal et al., 2007). The present study showed that although almost all OHCs in the basal turn were maintained under the protection of allicin, SCs and SGNs in the base were not protected effectively. P53 as well as pro-apoptosis positive staining in SCs and SGNs implied the occurrence of apoptosis, which may be the answer why mice in cisplatin + allicin group displayed hearing loss at 32 kHz.

### 4.2. Mechanism of allicin rescuing from cisplatin toxicity

Toxic drugs can activate a cascade of cell death genes, leading to cell death. An effective ear protection strategy may require intervention at multiple levels to regulate cell death and survival, therefore, it is essential to further understand the death pathway of the auditory cells. Although the mechanisms underlying cisplatin-induced apoptosis are not completely uncovered, it is believed to be involved in DNA damage, activation of p53, cell-cycle arrest and mitochondrial dysfunction (Jung and Lippard, 2007; Sugiyama et al., 1989). Indeed, in present study, TUNEL staining showed apoptosis of OHCs, SCs and SGNs in cisplatin group, whereas allicin reduced apoptosis in cisplatin + allicin group. To explore the potential mechanisms underlying the protection of allicin, two aspects were mainly discussed based on previous reports and our findings, one is anti-apoptosis, the other is antioxidant.

#### 4.2.1. Anti-apoptosis of allicin

A former study proved that apoptosis induced by cisplatin in inner ear cells was through the mitochondrial pathway (Garcia-Berrocal et al., 2007). The pro-apoptotic protein Bax could change the permeability of mitochondrial membranes and cause the release of cytochrome c. The Bcl-2 family of proteins prevent apoptosis by binding Bax protein. Imbalance between Bax and Bcl-2 released cytochrome c and activated caspase-9, finally activated the downstream caspase-3 to form cleaved-caspase-3 which induces apoptosis.

Consistently, our results of TEM showed that mitochondria in SGNs were extensively damaged in cisplatin group. However, allicin significantly reduced this damage induced by cisplatin. Furthermore, western blot and immunostaining in SGNs showed over expression of Bax, cleaved-caspase-9, cleaved-caspase-3 and down regulation of Bcl-2 after cisplatin treatment. The allicin down regulated expressions of Bax, cytochrome c, cleaved-caspase-9, cleaved-caspase-3 and raised expression of Bcl-2, suggested that allicin intervene in the mitochondrial-initiated cell death-signaling pathway to protect SGNs against cisplatin ototoxicity. The report that allicin played a neuroprotective effect by improving mitochondrial function (Zhu et al., 2012; Liu et al., 2015b) is consistent with our findings.

It is noted that the mitochondria in OHCs looked normal in all three groups including cisplatin treatment group, and cleaved-caspase-3 expression could not be detectable in OHCs of all groups (data not shown). It is speculated that apoptosis of OHCs induced by cisplatin may not be through the mitochondrial pathway.

P53 contributed to post-translational modifications that plays an important role on cell's function and stability (Kruse and Gu, 2009). Activation of p53 is a critical cellular response to DNA damage. A previous study showed that down regulation of p53 alleviated cisplatin-mediated cytotoxicity (Molitoris et al., 2009). In this study, p53 was expressed in the OHCs and SCs after cisplatin treatment, indicating that cisplatin might induce apoptosis of the OHCs and SCs through p53 pathway rather than the mitochondrial pathway. The results in our research were different from the previous study (Garcia-Berrocal et al., 2007), which might be related to the differences in dose of cisplatin and the species of animals. Interestingly, although allicin could significantly reduce the expression of p53 in OHCs, it could not obviously change the expression of p53 in SCs in the basal turn, suggesting SCs in the base were more sensitive and the damages were not remedied by allicin. Therefore, allicin could partly interfere with p53 apoptotic pathway to protect OHCs and SCs against cisplatin. The specific apoptotic pathway of p53 in OHCs and SCs induced by cisplatin need to be further studied.

#### 4.2.2. Antioxidation of allicin

Many studies have shown that cisplatin could cause ROS

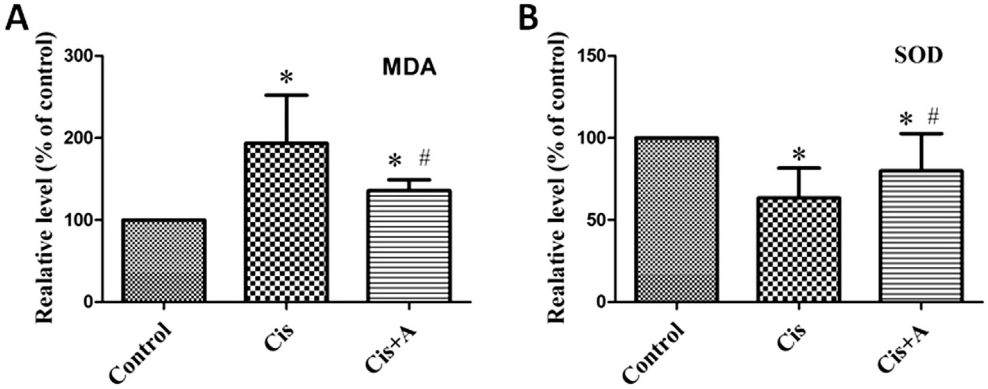


**Fig. 8.** Changes of MDA and SOD level in different groups. Compared with control group, cisplatin elevated MDA level (A) and decreased SOD level (B). Allicin protected cochlear by reversing these changes. *,#P < 0.05 by one-way ANOVA compared with the control (*) and Cis group (#). Control, 0.9% physiological saline; Cis, cisplatin; Cis + A, cisplatin + allicin. The data shown here were the mean ± SEM of three separate experiments.

production and damage inner ear cells through oxidative stress pathway (Rybak et al, 1995, 1997; Ravi et al., 1995). MDA is a biomarker of lipid peroxidation (Horton and Fairhurst, 1987). Superoxide dismutase (SOD) is one of the most powerful antioxidant of endogenous enzymes which protect against cisplatin-induced ototoxicity (Rybak et al., 1995; Campbell et al., 1996; Clerici, 1996). In the inner ear, it has been shown that increased antioxidant levels promoted hair cell survival meanwhile maintained function (Kawamoto et al., 2004). Our study found that cisplatin increased the level of MDA and decreased level of SOD in cochlear tissues, which was supported by previous studies (Garcia-Berrocal et al., 2007; Ravi et al., 1995; Salehi et al., 2014). Allicin reduced the level of MDA and increased the level of SOD in cochlear tissues, indicating that allicin could reduce the oxidative stress response to protect the cochlea from damages by cisplatin. Our results are in agreement with the previous studies (Zhu et al., 2012; Chen et al., 2014b; Li et al., 2012; Li et al., 2010).

In conclusion, to the date of our knowledge, this is the first study showing that allicin is effective in alleviating hearing loss casued by cisplatin, even though its protective effect is incomplete. Allicin protects SGNs from apoptosis induced by cisplatin through mitochondrial pathway while protects OHCs and SCs from apoptosis through p53 pathway. The present study provides the evidence that allicin, as a potential therapeutic drug, could prevent ototoxicity induced by cisplatin. However, there are also limitations. First, we did not test if allicin directly interacts with cisplatin or blocks its anti-tumor effect. Second, the specific apoptotic pathway of p53 in OHCs and SCs need to be further uncovered.

## Statement of interest

None.

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