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Allicin protects against cisplatin-induced vestibular dysfunction by inhibiting the apoptotic pathway



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ABSTRACT

Cisplatin is an anticancer drug that causes the impairment of inner ear function as side effects, including hearing loss and balance dysfunction. The purpose of this study was to investigate the effects of allicin against cisplatin-induced vestibular dysfunction in mice and to make clear the mechanism underlying the protective effects of allicin on oto-vestibulotoxicity. Mice intraperitoneally injected with cisplatin exhibited vestibular dysfunction in swimming test, which agreed with impairment in vestibule. However, these impairments were significantly prevented by pre-treatment with allicin. Allicin markedly reduced cisplatin-activated expression of cleaved-caspase-3 in hair cells and vascular layer cells of utricle, saccule and ampulla, but also decreased AIF nuclear translocation of hair cells in utricle, saccule and ampulla. These results showed that allicin played an effective role in protecting vestibular dysfunction induced by cisplatin via inhibiting caspase-dependent and caspase-independent apoptotic pathways. Therefore, allicin may be useful in preventing oto-vestibulotoxicity mediated by cisplatin.

1. Introduction

Cisplatin is a very effective and broad-spectrum anticancer drug that is commonly used in the treatment of various human cancers, including carcinoma of the head and neck (Kalcioğlu et al., 2005; Goncalves et al., 2013). However, the major side effects of cisplatin, including ototoxicity, nephrotoxicity and peripheral neuropathy, greatly impair a patient's quality of life (Barabas et al., 2008). Ototoxicity caused by cisplatin includes sensorineural hearing loss and vestibular dysfunction. Although the cochlear damages are largely known, the vestibular toxicity of cisplatin has received relatively little attention.

Some studies have elucidated the cisplatin cytotoxicity mechanisms were involved in DNA damage (Ries and Klastersky, 1986; Leibbrandt et al., 1995), forming reactive oxygen species (ROS) (Matsushima et al., 1998), mitochondrial dysfunction (Sugiyama et al., 1989) and caspase activation (Kaushal et al., 2001). A former study showed that apoptosis was activated in the vestibular cells handled with cisplatin (Zhang et al., 2003). However, the mechanisms underlying vestibular toxicity of

cisplatin are not completely clear.

Apoptosis-inducing factor (AIF), one of potentially toxic mitochondrial proteins, is confined to the mitochondrial intermembrane space in healthy cells (Cande et al., 2002a). Many studies have suggested that after the stimulation of several pro-apoptotic signals, AIF is released from mitochondria and then translocates to the nucleus where it binds to DNA, triggering caspase-independent cell death finally (Loeffler et al., 2001; Cande et al., 2002b, 2004). It has been reported that cisplatin could induce AIF-dependent apoptosis in renal cells and tumors (Seth et al., 2005; Liu et al., 2009; Jeong et al., 2014). However, no study of AIF concerning vestibular toxicity of cisplatin has been reported yet.

Unlike optional repair measures of cochlea such as hearing aids or cochlear implants, so far, there is no effective repair device for the loss of vestibular function (Wall et al., 2002). Therefore, the protection of vestibule is an important issue. Allicin, a major ingredient of fresh garlic extract, is a small lipophilic molecule that can freely permeate through the blood-brain barrier and accumulate up to a high level in the brain (Peng et al., 2002; Chauhan, 2003; Chung, 2006). Many

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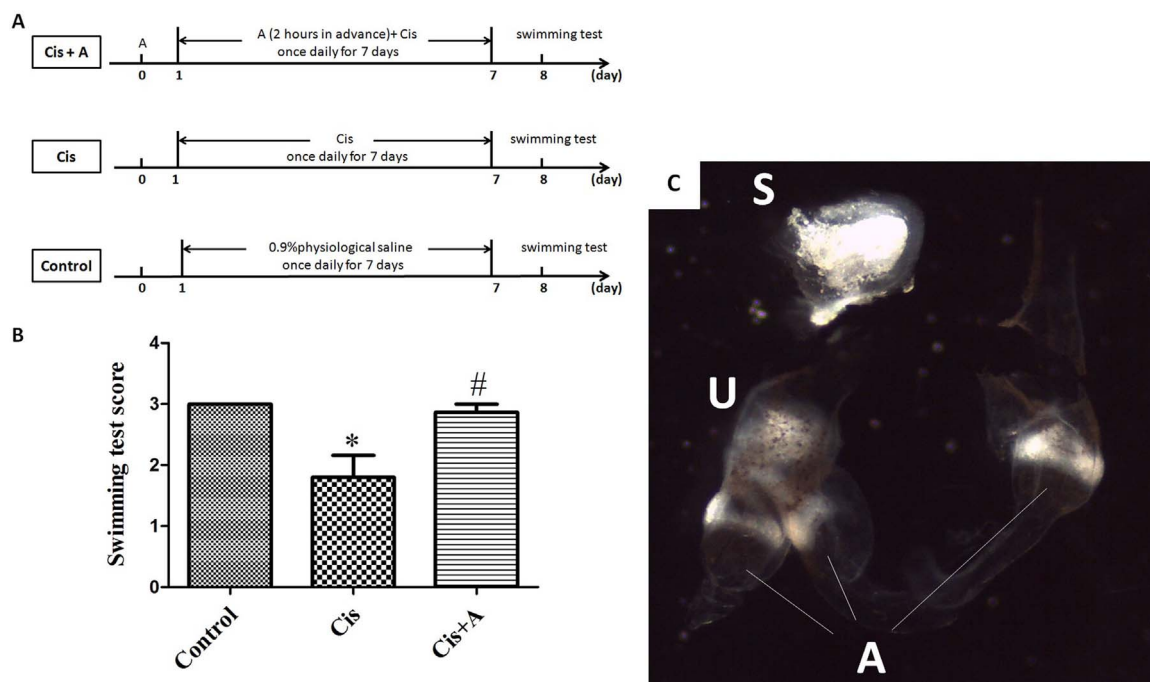


Fig. 1. Allicin protected against CDDP-induced vestibular dysfunction. (A) C57 mice were divided into three groups and given intraperitoneal injections of drugs as illustrated. (B) The average swimming test score after (post-8 day) allicin and after (post-7 day) cisplatin administration. Cisplatin treatment group (1.800 ± 0.3589 , $n=5$); Allicin group (2.866 ± 0.1340 , $n=5$); Control group (3.000 ± 0.0000 , $n=5$) * $P < 0.05$ vs Control group; # $P < 0.05$ vs Cis+A group; Control: 0.9% physiological saline, Cis: cisplatin, Cis+A: cisplatin+allicin. (each group, $n=5$). (C) The sketch map showed that the cristae ampulla, utricle and saccule were taken out respectively. A: ampulla, S: saccule, U: utricle.

studies have shown that allicin played a role in anti-microbial (Canizares et al., 2004; Cutler and Wilson, 2004) anti-tumor (Patya et al., 2004; Park et al., 2005), anti-oxidant (Borek, 2001; Liu et al., 2015), anti-inflammatory (Hodge et al., 2002; Lang et al., 2004) and anti-apoptosis (Zhang et al., 2008). Recently, it is demonstrated that allicin possess neuroprotective activity on traumatic or ischemic neuronal injury which is regulated by oxidative stress and apoptosis pathways (Chen et al., 2014; Zhou et al., 2014; Liu et al., 2015). A study also confirmed that allicin attenuated spinal cord ischemia–reperfusion injury through improving the function of mitochondria (Zhu et al., 2012). There are no results to date of protective effect of allicin on otovestibulotoxicity induced by cisplatin.

Therefore, the aim of the present study was to establish an animal model of the vestibular lesion induced by cisplatin, which was given a long time cisplatin treatment and was similar to that in clinical chemotherapy to determine whether cisplatin would cause damage to vestibule, whether AIF might participate in cisplatin-induced hair cell death of vestibule and whether allicin would be effective in alleviating vestibular damage mediated 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 cleaved-caspase-3 was acquired from Cell Signaling Technology (Beverly, MA, USA). Antibodies to Tuj 1 was acquired from Neuromics (Edina, USA). Antibody to AIF was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Click-iT Plus TUNEL assay kits were purchased from Life Technologies (Invitrogen, USA).

2.2. Experimental animals

All experiments were performed according to protocols approved by the Animal Care Committee of Shandong University, China, 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 ± 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 ($n=10$ 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. Groups 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 injection of cisplatin.

2.3. Swimming test

The swimming device for test was a rectangular water tank with 70 cm long and 40 cm wide, with room temperature water to the depth of 10 cm, the mice were placed in the center of the water tank and the body was parallel to the long axis of water tank, let go, to observe the free swimming posture. The swimmer who headed up and out the water with stable posture was recorded 3 scores. The swimmer who headed up and out the water, the posture is not steady, was recorded 2 scores. The swimmer whose head could not upward or could not expose to the surface of the water was recorded 0 score, the mouse must be

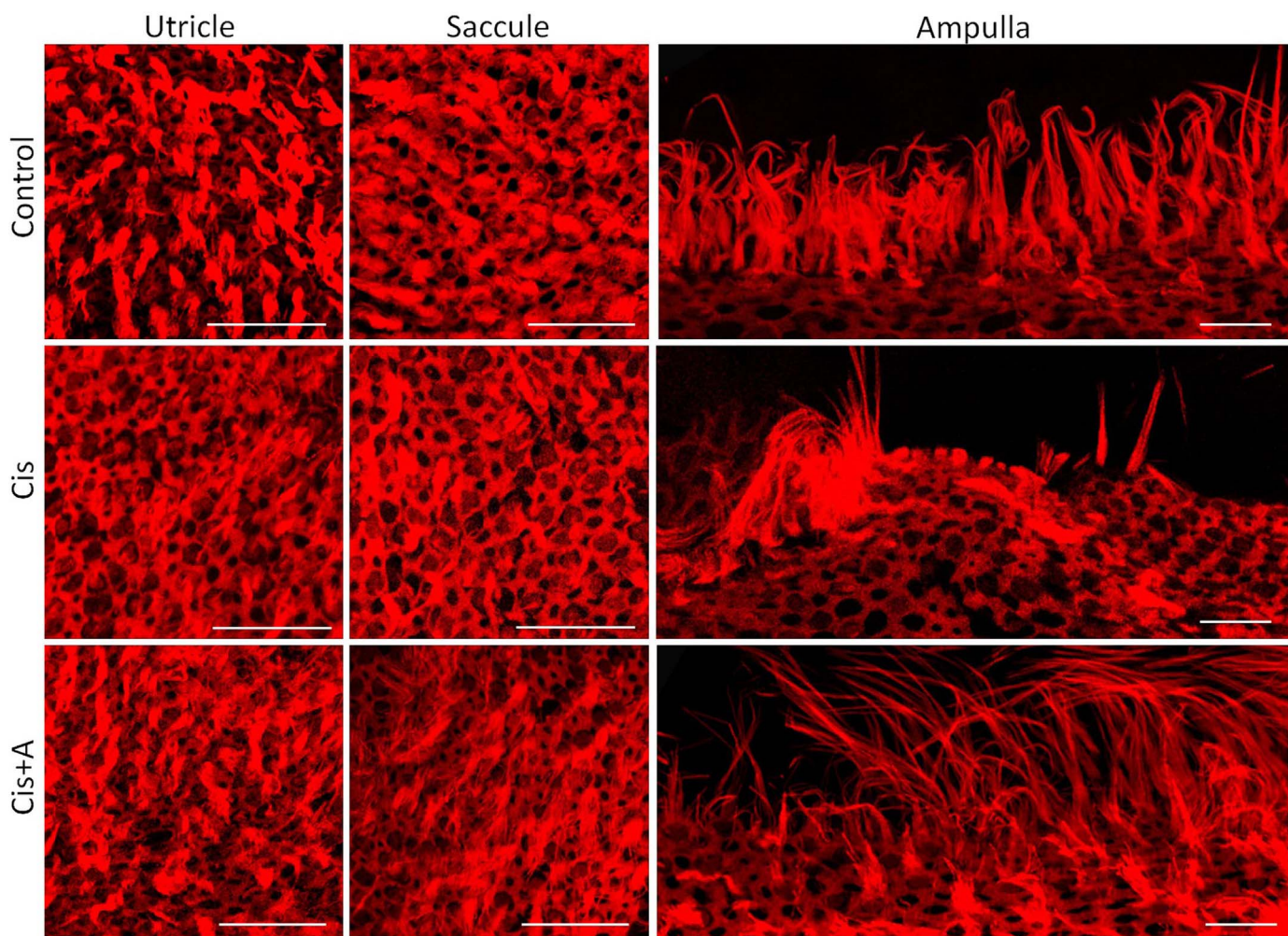


Fig. 2. Effects of alliin against cisplatin-induced the loss of hair bundles of hair cells in vestibule. The phalloidin staining showed that after (post-7 day) cisplatin administration, the utricule, saccule and ampulla in the cisplatin group showed an obviously reduced number of hair bundles of hair cells with an abnormal appearance compared with the control group; alliin could preserve hair bundles of hair cells with normal appearance and a high density. Control: 0.9% physiological saline, Cis:cisplatin, Cis+A:cisplatin+alliin. (each group, n=3). Scale bars: 25 μ m.

immediately rescued from the water to avoid drowning. 5 mice were randomly selected in each group for swimming test, each mouse was tested 3 times, 1 min each time, each time interval of 10 min. Test alternately, preventing excessive fatigue which might effect the results of test. Counted the average and performed statistic analysis. Tests were conducted at room temperature.

2.4. Sample collection

Seven days after drug administration, C57 mice were anesthetized with lethal doses of chloral hydrate and the right inner ear tissues were harvested and perfused with 4% paraformaldehyde in PBS and further fixed with 4% paraformaldehyde for 24 h at room temperature. After decalcification with 0.1 M EDTA for 2 days and an overnight incubation in 20% sucrose. The inner ear tissues were post-fixed in dry ice for frozen section in TUNEL staining (each group n=3). The left inner ear tissues were harvested and immersed in the pre-cold Hanks' solution, dissected under a dissecting microscope, removed the vestibular bone part, took the ampullas, utricles and saccules out respectively and removed the otolithic membrane on the macula (Fig. 1C). The ampullas, utricles and saccules were towared positive, adhered on the small slide of the pre-coated with celltack and fixed in 4% paraformaldehyde at room temperature for immunofluorescence staining (each group n=3).

2.5. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nickend-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).

2.6. Immunofluorescence staining

Each sample was washed triple with PBS, followed by the fixation with 4% paraformaldehyde and permeabilization with 1% TritonX-100 in PBS (Sigma), sections were immersed in blocking solution (0.1% TritonX-100, 8% donkeyserum, 1% bovine serum albumin, and 0.02% sodium azide in PBS) at room temperature for 1 h. Then, samples were incubated with diferent primary antibodies: cleaved- caspase-3 (1:400), AIF (1:100) and Tuj-1 (1:1000, Neuromics), diluted in blocking solution, respectively, at 4 °C overnight. 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) in 0.1% TritonX-100% and 1% BSA in PBS at

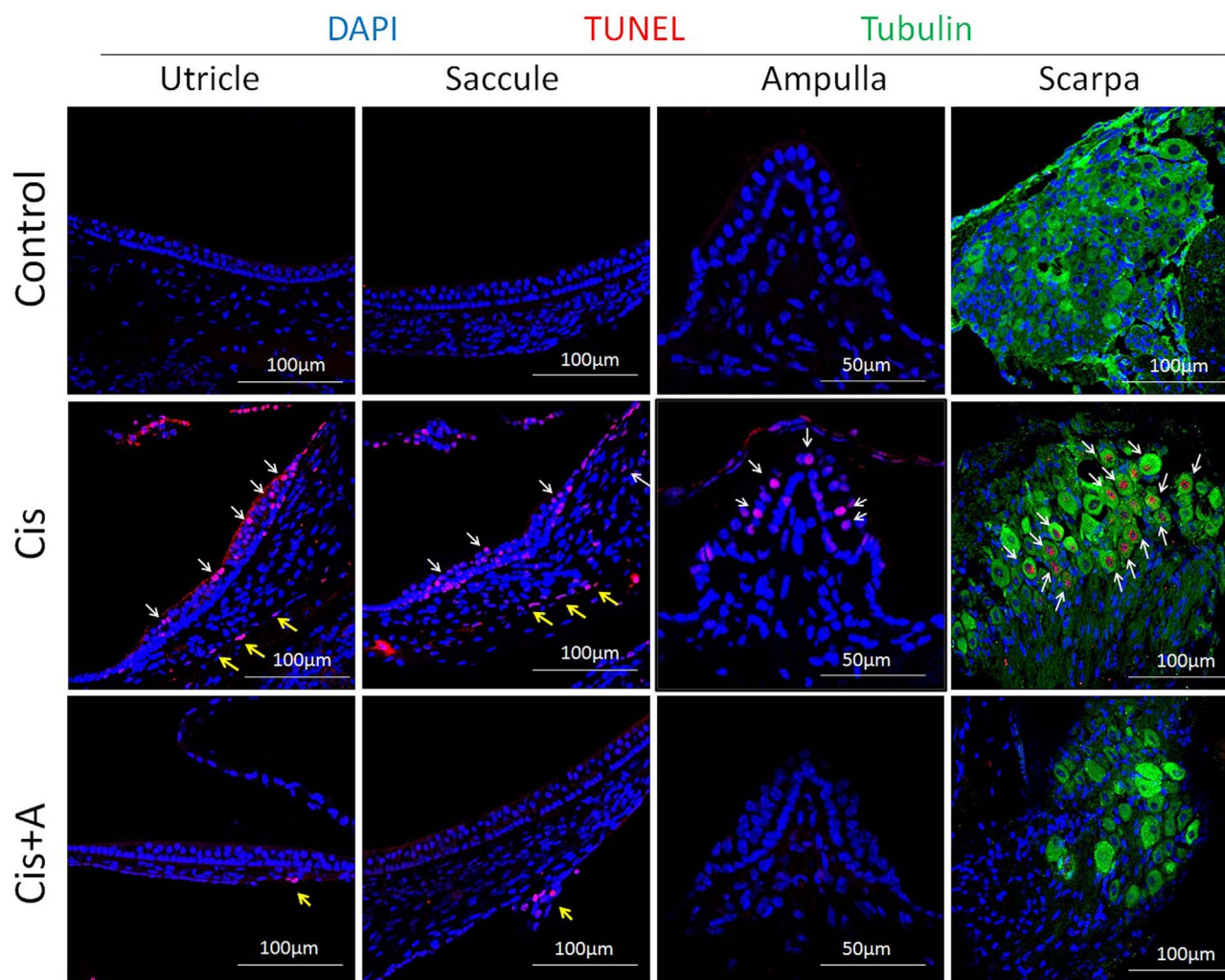


Fig. 3. Allixin protected vestibular cells from apoptotic death induced by cisplatin. After (post-7 day) cisplatin administration, positive TUNEL staining were found in neurons of vestibular ganglion (Scarpa), mesenchymal cells, hair cells and supporting cells in utricle, saccule and ampulla in cisplatin group. Allixin could significantly reduce TUNEL staining induced by cisplatin. There was no staining of TUNEL staining in vestibular cells in control group. Control: 0.9% physiological saline, Cis:cisplatin, Cis+A:cisplatin+allixin. (each group, n=3). White arrows indicate TUNEL staining of nuclear in vestibular ganglion (Scarpa), hair cells and supporting cells in utricle, saccule and ampulla. Yellow arrows indicate TUNEL staining of nuclear in mesenchymal cells. Scale bars: 100 μ m or 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

room temperature in darkness for 1 h. After the final wash with PBS, specimens were mounted on slides with anti-fade mounting media and imaged on a Leica confocal microscope (Leica, Germany).

2.7. 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 \pm S.E.M. Statistical analysis were performed by one-way analysis of variance (ANOVA) followed by the Newman Keuls multiple-comparison post hoc test and Chi-square test. P value < 0.05 or P value < 0.017 was considered statistically significant (Chi-square test was used in AIF nuclear translocation ratio analysis, 3 groups, comparison between two groups, so P < 0.05 was divided by 3).

3. Results

3.1. Allixin protected against cisplatin-induced vestibular dysfunction

In swimming test, vestibular function of C57 mice was damaged in the cisplatin group (1.800 ± 0.3589 , n=5) compared to control

group (3.000 ± 0.0000 , n=5), manifesting that the swimming posture was instability and/or head was not exposed to the surface of water. Allixin could significantly improve the vestibular function of C57 mice, manifesting in steady swimming posture and exposed head out of the water. The swimming test score showed significant difference between the cisplatin treatment group and allixin group (2.866 ± 0.1340 , n=5) (Fig. 1. B).

3.2. Effects of allixin against cisplatin-mediated the loss of hair bundles of hair cells in vestibule

After 7 days of cisplatin treatment, the utricle, saccule and ampulla in the cisplatin group showed an obviously reduced number of hair bundles of hair cells with an uneven distribution and abnormal appearance compared with the control group; however, hair bundles of hair cells in the cisplatin+allixin group were well-preserved with normal appearance and a high density (Fig. 2).

3.3. Allixin protected vestibular cells from apoptosis induced by cisplatin

There was no staining of TUNEL staining in vestibular cells in

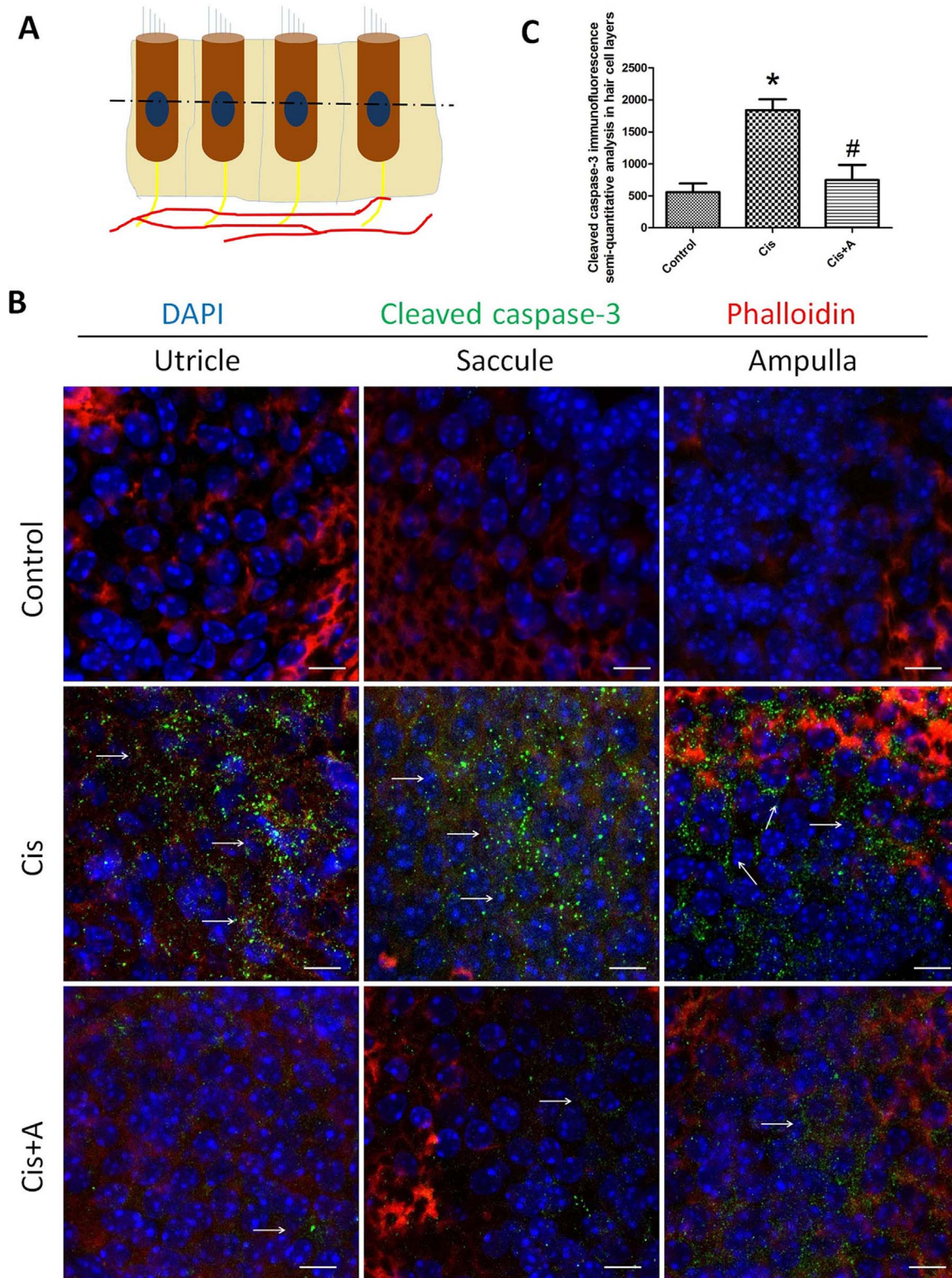


Fig. 4. Effect of Allicin on CDDP-induced apoptosis via inhibiting the expression of cleaved-caspase-3 in vestibular hair cells. The sketch map of immunostaining of hair cells layer in vestibule. (B) There was no staining of cleaved-caspase-3 in control group. After (post-7 day) cisplatin administration, the cytoplasm of hair cells in utricle, saccule and ampulla were positive by cleaved-caspase-3 staining in cisplatin group and allicin could significantly decrease the expression of cleaved-caspase-3 induced by cisplatin. Control: 0.9% physiological saline, Cis: cisplatin, Cis+A: cisplatin+allicin. each group, n=3. Arrows indicate cleaved-caspase-3 staining of cytoplasm. Scale bars: 10 μ m. (C) Cleaved-caspase-3 immunofluorescence semi-quantitative analysis in hair cells layers. The fluorescence density of cleaved-caspase-3 in hair cells layers in cisplatin group was significantly higher than that in control group, while allicin significantly reduced the fluorescence density of cleaved-caspase-3 in cisplatin+allicin group. *, #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).

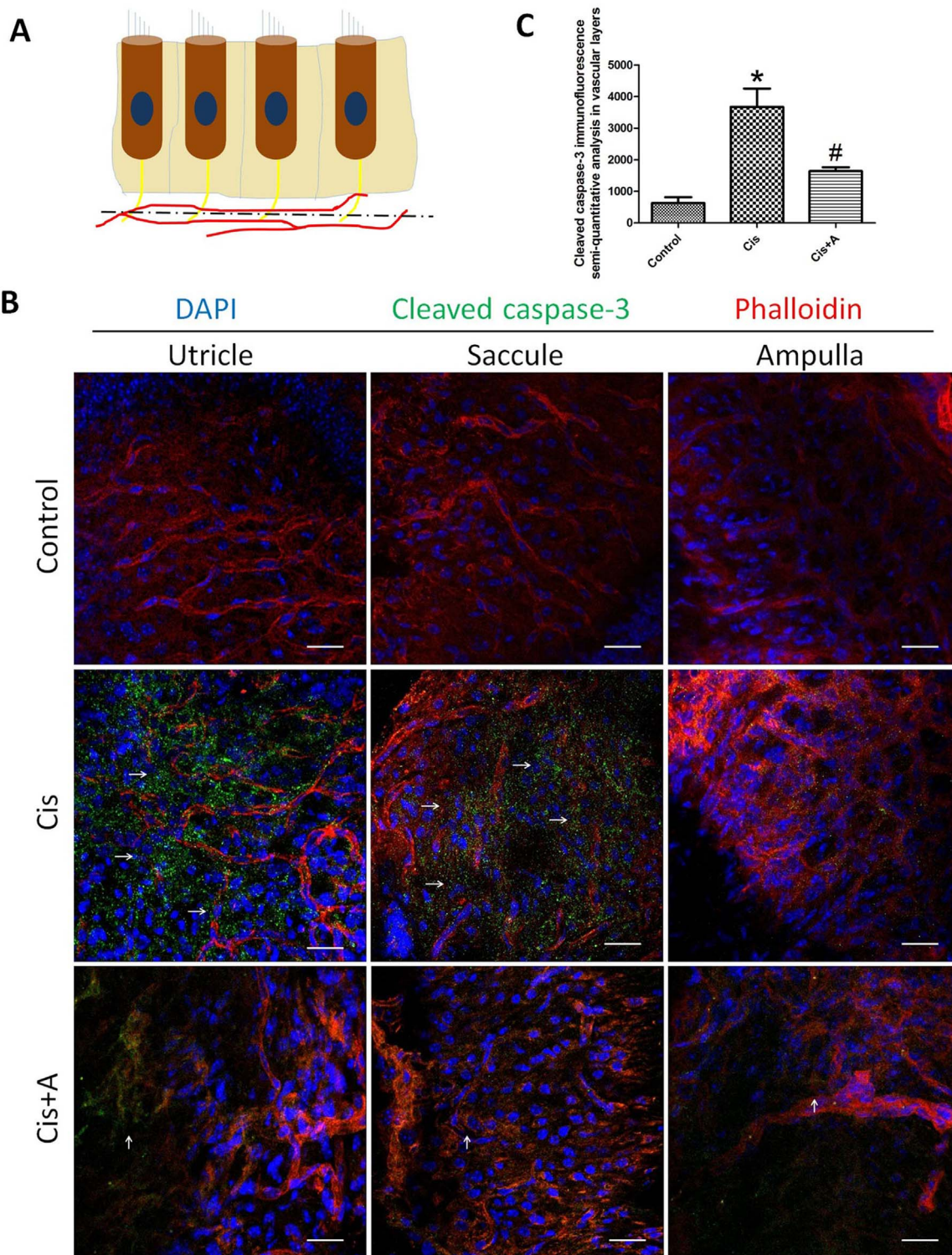


Fig. 5. Effect of Allicin on cisplatin-induced apoptosis via inhibiting the expression of cleaved-caspase-3 in vascular layer cells. (A) The sketch map of immunostaining of vascular layer in vestibule. (B) There was no staining of cleaved-caspase-3 in control group. After (post-7 day) cisplatin administration, the cytoplasm of vascular layer cells in utricule, sacculle and ampulla were positive by cleaved-caspase-3 staining in cisplatin group and allicin could significantly decrease the expression of cleaved-caspase-3 induced by cisplatin. Control: 0.9% physiological saline, Cis: cisplatin, Cis+A: cisplatin+allicin. each group, n=3. Arrows indicate cleaved-caspase-3 staining of cytoplasm. Scale bars: 10 μ m. (C) Cleaved-caspase-3 immunofluorescence semi-quantitative analysis in vascular cells layers. The fluorescence density of cleaved-caspase-3 in vascular cells layers in cisplatin group was significantly higher than that in control group, while allicin significantly reduced the fluorescence density of cleaved-caspase-3 in cisplatin+allicin group. *, #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).

control group. Following 7 days of cisplatin treatment, positive TUNEL staining were found in neurons of vestibular ganglion, mesenchymal cells, hair cells and supporting cells in utricule, sacculle and ampulla in cisplatin group. However, no TUNEL staining could be observed in

vestibular ganglion, hair cells and supporting cells in allicin treatment group. Only some mesenchymal cells were marked by TUNEL staining (Fig. 3).

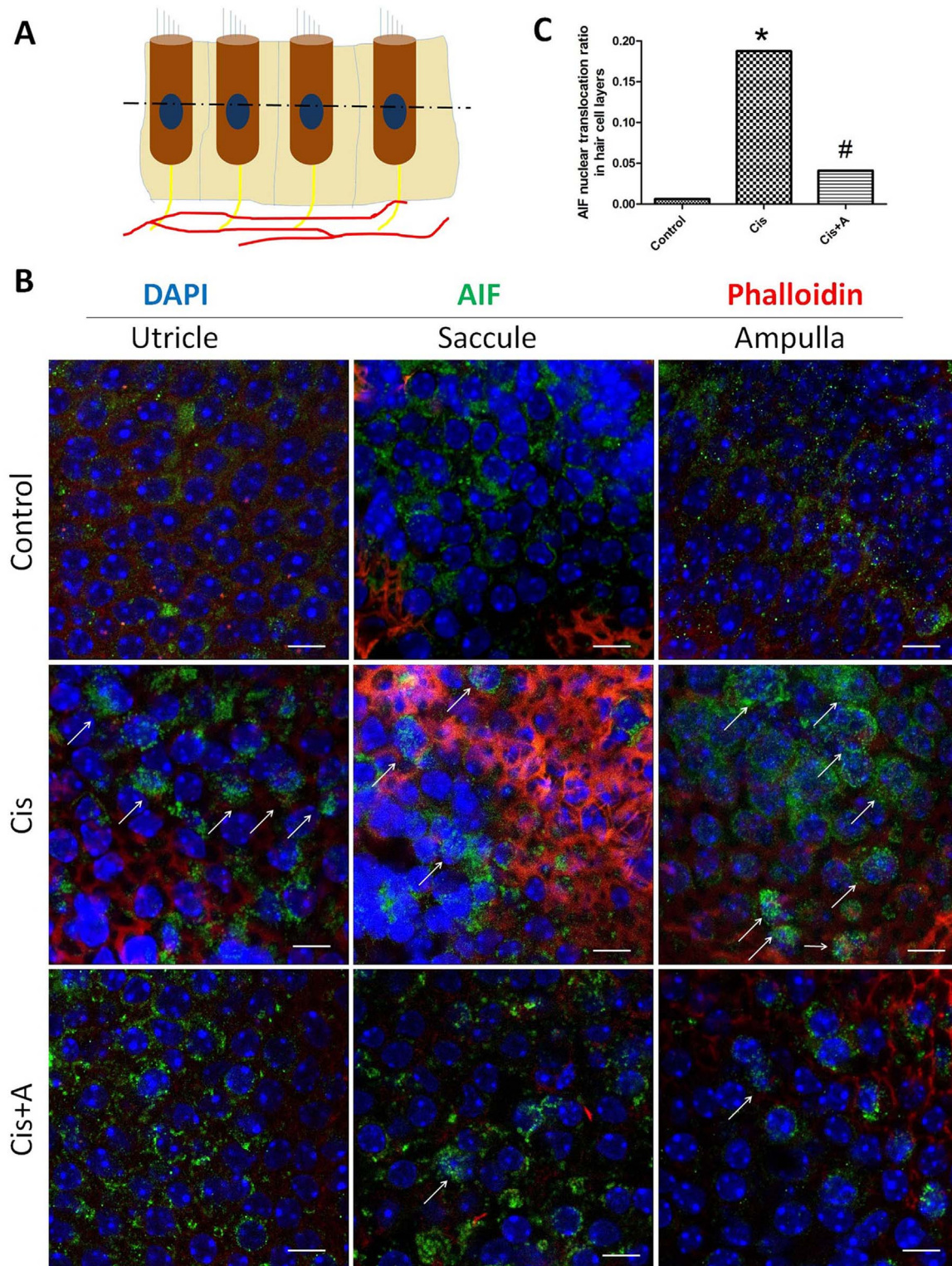


Fig. 6. Effect of Allicin on cisplatin-induced apoptosis via inhibiting the expression of AIF in vestibular hair cells. (A) The sketch map of immunostaining of hair cells layer in vestibule. (B) AIF was a weak distribution surrounding the nucleus and no distribution within the nucleus exhibited in control group. After (post-7 day) cisplatin administration, AIF was detected within the nuclear region in hair cells of utricule, sacculle and ampulla. Allicin could significantly reduce the nuclear translocation of AIF induced by cisplatin. Control: 0.9% physiological saline, Cis:cisplatin, Cis+A:cisplatin+allicin. each group, n=3. Arrows indicate AIF staining of nuclear. Scale bars: 10 μ m. (C) AIF nuclear translocation ratio in hair cell layers. The nuclear translocation ratio of AIF in hair cells layers in cisplatin group was significantly higher than that in control group, while allicin significantly reduced the AIF nuclear translocation ratio in cisplatin+allicin group. *, # $P < 0.017$ by Chi-square test compared with the control (*) and cisplatin group (#). Control: 0.9% physiological saline; Cis, cisplatin; Cis+A, cisplatin+allicin (each group, n=3).

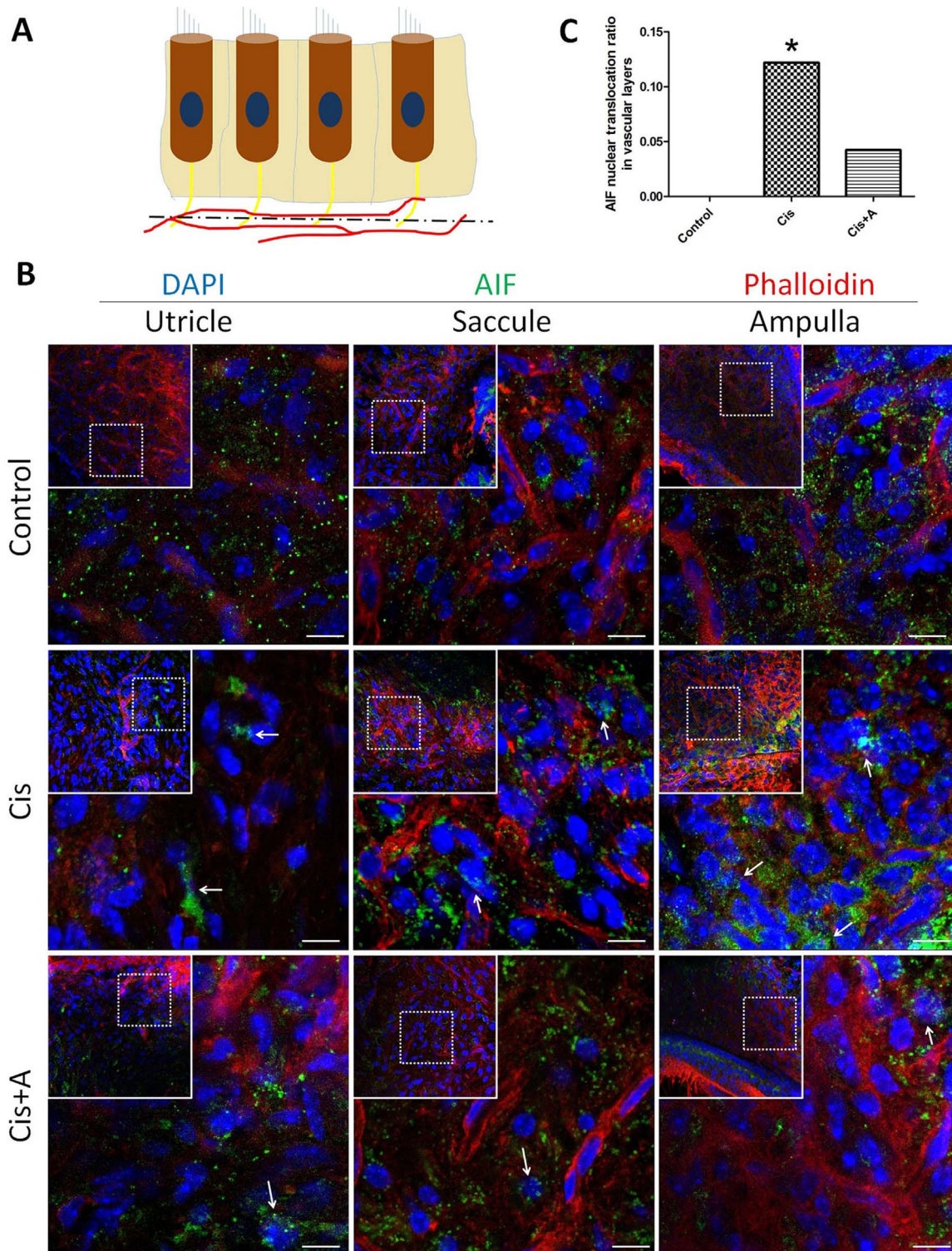


Fig. 7. Effect of Allicin on cisplatin-induced apoptosis via inhibiting the expression of AIF in vascular layer cells. (A) The sketch map of immunostaining of vascular layer in vestibule. (B) AIF was a weak distribution outside the nucleus and no distribution within the nucleus exhibited in control group. After (post-7 day) cisplatin administration, AIF was detected within the nuclear region in vascular layer cells of utricule, saccule and ampulla. Allicin could significantly reduce the nuclear translocation of AIF induced by cisplatin. Control: 0.9% physiological saline, Cis:cisplatin, Cis+A:cisplatin+allicin. each group, n=3. Arrows indicate AIF staining of nuclear. Scale bars: 10 μ m. (C) AIF nuclear translocation ratio in vascular cells layers. The nuclear translocation ratio of AIF in vascular cells layers in cisplatin group was significantly higher than that in control group, while allicin didn't significantly reduced the AIF nuclear translocation ratio in cisplatin+allicin group. * $P < 0.017$ by Chi-square test compared with the control (*). Control: 0.9% physiological saline; Cis, cisplatin; Cis +A, cisplatin+allicin (each group, n=3).

3.4. Effect of allicin on cisplatin-induced apoptosis via inhibition of the caspase-dependent apoptotic pathways

Immunofluorescence results showed that there was no staining of

cleaved-caspase-3 in control group. However, in cisplatin treatment group, positive staining of cleaved-caspase-3 could be found in the cytoplasm of hair cells (Fig. 4B) and vascular layer cells (Fig. 5B) in utricule, saccule and ampulla. In cisplatin+allicin group, the expression

of cleaved-caspase-3 was significantly decreased both in the cytoplasm of hair cells and vascular layer cells. The fluorescence density of cleaved-caspase-3 in hair cells layers and vascular layer cells in cisplatin group were significantly higher than that in control group, while allicin significantly reduced the fluorescence density of cleaved-caspase-3 in hair cells layers and vascular layer cells in cisplatin+allicin group (Figs. 4C and 5C).

3.5. Effect of Allicin on cisplatin-induced apoptosis via inhibition of the caspase-independent apoptotic pathways

In control group, AIF was distributed surrounding the nucleus in the cytoplasm and no distribution within the nucleus. After 7 days of cisplatin treatment, a strong fluorescence of AIF was detected within the nuclear region both in hair cells (Fig. 6B) and vascular layer cells (Fig. 7B). The nuclear translocation ratio of AIF in hair cells layers and in vascular cells layers in cisplatin group were significantly higher than that in control group, while allicin significantly reduced the AIF nuclear translocation ratio in hair cells layers and allicin didn't significantly reduced the AIF nuclear translocation ratio in vascular cells layers in cisplatin+allicin group. The result revealed the translocation of AIF from mitochondria to the nucleus in hair cells and vascular layer cells of utricle, saccule and ampulla. In cisplatin+allicin group, allicin significantly reduced the nuclear translocation of AIF in hair cells layers mediated by cisplatin.

4. Discussion

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

So far, vestibular toxicity induced by cisplatin is controversial. On the one hand, some animal studies showed that cisplatin could lead to vestibular dysfunction (Sergi et al., 2003, 2004; Lo et al., 2015; Ma et al., 2015). Even low dose of cisplatin can cause the damage of vestibular hair cells in guinea pigs (Nakayama, 1992; Nakayama et al., 1996). Vestibular dysfunction was also observed in some patients treated by cisplatin (Black et al., 1982). On the contrary, another study showed that cisplatin caused outer hair cell damage in cochlea, but without injuring the vestibular end-organ (Laurell and Bagger-Sjoberg, 1991). Myers's study reported that there was no clear evidence of the vestibular toxicity in patients who receiving cisplatin chemotherapy (Myers et al., 1993). Our present study found that intraperitoneal injection of cisplatin could cause damage to the vestibular function of mice. From the viewpoint of morphology, cisplatin obviously damaged the hair bundles of vestibular hair cells with an abnormal appearance. These results were consistent with the previous studies (Sergi et al., 2003, 2004; Tian et al., 2013; Ma et al., 2015). However, pre-treatment with allicin could significantly prevent these impairments. The above results proved the protective effect of allicin on the vestibular function and morphology against cisplatin.

4.2. Mechanisms underlying allicin protecting against cisplatin-mediated vestibular toxicity

The TUNEL staining confirmed that cisplatin could cause apoptosis in neurons of the vestibular ganglion, mesenchymal cells, hair cells and supporting cells in utricle, saccule and ampulla. However, the mechanisms underlying vestibular toxicity caused by cisplatin are still unclear.

Previous studies found that cisplatin treatment could mediate apoptosis by activating caspase-2, -8, -9, -3 (Kaushal et al., 2001; Watanabe et al., 2001) and placed caspases (a family of cysteine-aspartate-specific proteases) as a pivotal role in causing cell death. Of which caspase-3 plays a dominant role in the caspase cascade characteristic of the apoptotic pathway, which is cleaved into 17 kDa

and 12 kDa subunits when it is triggered. This study found that the expression of cleaved-caspase-3 was enhanced by cisplatin, which is supported by the previous studies that cisplatin induced caspase-dependent apoptosis (Watanabe et al., 2001). Allicin could significantly reduce the expression of cleaved-caspase-3, suggesting that allicin could prevent to some extent caspase-dependent apoptosis induced by cisplatin in vestibular hair cells and vestibular vascular layer cells.

There are in vitro and in vivo studies documenting that cisplatin can inhibit mitochondrial ATPase activities in vestibular tissues (Cheng et al., 2005, 2006). Therefore, it is speculated that mitochondrial damage might have an important role in the pathogenesis of vestibular dysfunction induced by cisplatin. In this study we found that AIF was nuclear translocation in vestibular hair cells and vestibular vascular layer cells after treatment by cisplatin, which was supported by previous studies. Allicin could significantly reduce the AIF nuclear translocation ratio mediated by cisplatin, and obviously protect mitochondrion from injuring by cisplatin in vestibular hair cells. Interestingly, allicin didn't significantly reduce the AIF nuclear translocation ratio mediated by cisplatin in vestibular vascular layer cells, which needs to be further explored.

Briefly, the defects in vestibular function and morphology induced by cisplatin could be rescued by allicin treatment. Allicin could significantly reduce the expression of cleaved-caspase-3 and AIF nuclear translocation mediated by cisplatin, obviously protect mitochondrion from injuring by cisplatin in vestibular hair cells through regulating both the caspase-dependent and AIF-mediated caspase-independent apoptotic pathways and allicin obviously protect vascular layer cells through the caspase-dependent and apoptotic pathways.

5. Conclusion

To the best of our knowledge, this is the first study showing that allicin might inhibit cisplatin-induced apoptosis in vestibular cells and vestibular ganglion cells, protect the vestibular function of mice. The anti-apoptotic properties of allicin will provide a new therapeutic method for the treatment of vestibulotoxicity 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 apoptotic pathways of vestibular ganglion cells and vestibular mesenchymal cells need further study.

Statement of interest

None.

Funding statement

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