Am J Transl Res 2018;10(11):3650-3663
www.ajtr.org /ISSN:1943-8141/AJTR0078275

Original Article

Vincamine exerts protective effect on spiral ganglion neurons in endolymphatic hydrops guinea pig models

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Received April 21, 2018; Accepted October 16, 2018; Epub November 15, 2018; Published November 30, 2018

Abstract: The aim of this study was to investigate the protective effects of vincamine in endolymphatic hydrops (ELH). After ELH guinea pigs treated by vincamine, the concentration of VAP in plasma, and the levels of cAMP, MDA, SOD, GSH-Px in right cochlea were measured using spectrophotometric method. The V2R, NMDAR1, p-NMDAR1, AQP2, p-AQP2, caspase3/9 and c-caspase3/9 expressions in right cochlea were detected using western blot analysis. The cochlear hydrops degree and SGNs density were evaluated by hemotoxylin and eosin staining (HE) test. Normal hearing and vestibular function were warranted by the tests of auditory brainstem response (ABR) and electronystagmography (ENG). After glutamate-injured SGNs treated with vincamine, the MDA, SOD GSH-Px, NGF, BDNF, NT3, NT4 and Trks levels were measured. Meanwhile, the Bcl2, Bax, NMDAR1, p-NMDAR1, PI3K, p-PI3K, Akt, p-Akt, caspase3/9 and cleaved-caspase3/9 expression levels were detected. Furthermore, the viability, apoptosis and necrosis of SGNs were tested by MTT and Hoechst/PI staining methods. The results indicated that vincamine could significantly inhibit the expression levels of cAMP, MDA, V2R, p-NMDAR1, p-AQP2 and c-caspase-3/-9 in cochlea, alleviate the cochlear hydrops degree, regulate the audiological and vestibular dysfunctions. The SGNs density, SOD and GSH-Px levels were also increased by vincamine. In vincamine-treating groups, the MDA, Bax, p-NMDAR1, and c-caspase3/9 levels were observably decreased, while SGNs survival, SOD, GSH, NGF, BDNF, NT3, NT4, Trks, Bcl2, p-PI3K, p-Akt expressions were improved. The present study indicated a novel use of vincamine in suppressing ELH formation by down-regulating the VAP/AQP2 signaling pathway. It also manifested that vincamine exerted protective effects on hearing via improving neurotrophin-dependent PI3K/Akt signaling pathway in SGNs.

Keywords: Meniere's disease, endolymphatic hydrops, VAP/AQP2, PI3K/Akt, vincamine

Introduction

Meniere's disease (MD) is a clinical disorder that is characterized by the episodes, including spontaneous vertigo, hearing loss, tinnitus and fullness in the ear. MD is a multifactorial disease, the familial inheritance, autoimmune reaction and other environmental factors may probably determine its occurrence [1]. Endolymphatic hydrops (ELH) is a pathological marker mostly seen in MD [2]. ELH is caused by the incoordination of the inner ear water homeostasis, which possibly depends on the regulation of vasopressin (VAP). Human and animal studies have demonstrated that the endolymphatic sacs of MD had significantly higher levels of VAP2 receptor (V2R), which suggested that MD patients may be more sensitive to VAP. Furthermore, chronic administration of AVP has been demonstrated to induce ELH formation [3, 4]. Recent evidence also suggests that ELH has a causal relationship with MD [5]. In clinical cases, the vertigo episodes improved accompanied with the hearing function deteriorated are frequently observed in MD patients. Numerous studies have revealed that the vertigo was attributed to ELH, whether idiopathic or secondary [6]. Meanwhile, examinations of temporal bones have also showed a pattern of spiral ganglion neurons (SGNs) degeneration, started at the apex of the cochlea in MD patients. Damage to SGNs rather than damage to sensory hair cells is directly associated with the ELH mediated stress in hearing loss [7]. These observations are suggestive of the possibility that ELH may play a role in the pathogenesis of MD-related vertigo attacks and hearing loss. Particularly, MD is more common than systemic lupus erythematosus and multiple sclerosis, with the prevalence reached to 0.19%. The

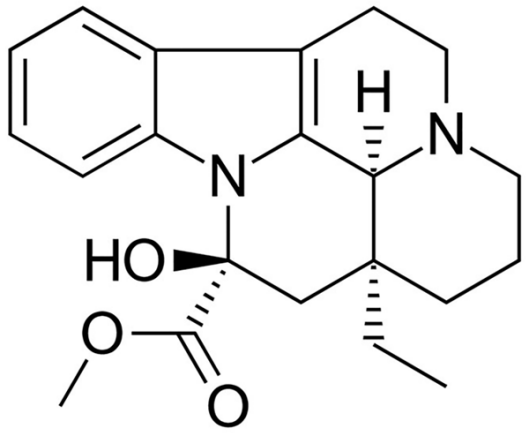

Figure 1. The chemical structure of vincamine. Molecular formula: $C_{21}H_{26}N_2O_3$. Molecular weight: 354.44 g/mol.

prevalence of MD is elevated dramatically with increasing age in the United States, which significantly affects the elderly life quality without any effective treatments [8, 9]. Thus, it is important to explore effectual strategies on eliminating the ELH and related complications in MD.

Herbal medicines have been proven to be a major source of novel agents with various pharmaceutical activities. Vinca minor is a species of flowering plant, which mainly distributes in Eastern Europe and North Africa, has been used as anti-aging drugs (or nutritional supplements) for thousands of years [10]. Vincamine (**Figure 1**) is an indole alkaloid found in the leaves of Vinca minor [11]. It is widely prescribed in Europe and other regions for the treatment of cerebrovascular diseases, cognitive disorders, and certain types of stroke [12]. Further studies have indicated that vincamine was a cerebral metabolic enhancer in treating ischemia and hypoxia, via its effect on peripheral vasodilation, ATP production, and efficient utilization of glucose and oxygen [13, 14]. Vincamine could also enhance dopaminergic, serotonergic and noradrenergic functions, and improve neural protection [15]. Previous clinical research shows the tinnitus and vertigo are decreased by vincamine in old patients with neuropsychiatric symptoms [16], which suggests the potential activities of vincamine in inner ear. Based on its efficacy and safety, we hypothesize that vincamine would improve the cochlear functions by treating both ELH and SGNs injury. In this study, to develop effective treatment for the patients with endolymphatic hydrops, we aim to investigate the efficacy of vincamine in ELH guinea pig models and glutamate injured SGN cells. Overall, these findings might provide valuable information for drug development or potential strategies against ELH-associated MD.

Materials and methods

Animal procedures

All experiments were performed using albino guinea pigs (350 ± 50 g), which were kindly provided by the Experimental Animal Center of the Chinese Academy of Science (Shanghai, China) and maintained in a pathogen-free environment at the Animal Center of Shanghai University of Traditional Chinese Medicine. Normal hearing and vestibular function were warranted by the tests of auditory brainstem response (ABR) and electronystagmography (ENG) before and 6, 12 weeks after the surgery. Subsequently, guinea pigs were anesthetized with a combination, including (16.7 mg/kg, i.p.), xylazine (3.5 mg/kg, i.m.) and ketamine (17.3 mg/kg, i.m.). Unilateral ELH was induced on the right side as described by Andrews [17]. In brief, extradural posterior cranial fossa approach was carried out. After the occipital bone drilled, the sigmoid sinus, transverse sinus, and dura over the cerebellum were exposed. The endolymphatic duct and sac were identified by pulling the sigmoid sinus slightly medially and inferiorly, and obliterated with bone pieces, then interposed and putted it forward between sigmoid sinus and operculum. Meanwhile, the sham-operated guinea pigs received sham operation on the right ear by exposing the endolymphatic duct and sac, without any destructive operation. Finally, postoperative injection with ceftriaxone (30 mg/kg, i.p.) was applied for 3 days. 6 weeks after surgery, 40 model guinea pigs (either gender, with obvious hearing and vestibular dysfunctions) were tenderly screened and allocated into 4 groups, and then received intragastric administration of saline and/or vincamine (10 or 30 mg/kg/d) and/or tolvaptan (10 mg/kg/d) for 6 weeks. 10 sham-operated guinea pigs were equally received saline. Furthermore, after the audiological and nystagmus tests, blood was collected from lateral saphenous and centrifuged (3,000 r/min, 15 min, 4°C) immediately, then the supernatants were stored at -80°C. All animals were sacrificed under deep anesthesia, and then cochleas form the right ear were dissected. Fractions were flash-frozen in liquid nitrogen and stored

in separate tubes at -80°C, the remaining parts were fully submerged in 10% buffered formalin, decalcified and embedded in celloidin. After hardening process, the specimens were cut along the mid-modiolar plane into 14-μm sections. The sections were slide mounted and stained with hematoxlyin and eosin for light microscopy. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai University of Traditional Chinese Medicine.

Hearing and vestibular function tests

ABR threshold was recorded using Nicolet Spirit evoked potential system (Nicolet Spirit evoked potential system, Madison, Wisconsin, USA). Briefly, guinea pigs were intraperitoneally injected with sodium pentobarbital (35 mg/kg) in audiometric room. The reference electrode was subcutaneously placed at auricular edge, accompanied with active electrode implanted at vertex and grounding electrode implanted at the nose. The ipsilateral ear was stimulated with clicks (75 ms interval) through a TDH-39 earphone (Telephonics, Chester, Cheshire, UK), when another ear was plugged. Finally, evoked potential was filtered with a bandpass (0.1~3 kHz), averaged for 128 times. The audiological test on guinea pigs was performed before and 6, 12 weeks after the surgery.

Nystagmus was examined using RM-6100 mini polygraph (Nikon Kohden, Tokyo, Japan). Firstly, the electrodes were covered with conductive paste. The depilation and degreasing were carried out around the eyes and vertex, with two disk electrodes placed on canthus of each eye, and one grounding electrode placed on vertex. Then the guinea pig was fixed on Fod-03 rotary chair (Daiichi Medical, Tokyo, Japan), while its head was pitched downward 48 degrees. Finally, the sinusoidal rotation was set (180 degree, 0.1 Hz, 6 seconds per circle). The vestibular function test on guinea pigs was performed in darkroom before and 6, 12 weeks after the surgery.

ELH degree and SGNs density examinations

Subsequently, the specimens were decalcified in ethylenediamine tetraacetic acid (EDTA). After 7 days, these specimens were dehydrated in a graded ethanol series, then embedded in paraffin. The prepared blocks were horizontally cut parallel to the axis of the modiolus into 5 μm sections. The sections were stained with hematoxylin and eosin for observation under a light microscope. The changes of endolymphatic space were observed by investigators in a blind fashion. Briefly, the second turn was selected in the mid-modiolar section of each cochlea for its excellent reliability, and images were captured with a digital camera (Nikon Cool PIX990, Tokyo, Japan). The area of the scala media (SM) and scala vestibule (SV) of bilateral modiolus were measured as described by Klis [18]. The ELH degree was calculated according to the ratio of SM area to SM + SV area.

Spiral ganglion neurons were counted as described by Yagi [19]. Briefly, three non-adjacent sections were selected from each cochlea. The operator used bony borders of Rosenthal's canal to define the detection regions of cochlea, and SGNs were counted in these regions. The area of detection region was measured using Image-pro plus6.0 software (Media Cybernetics, Rockville, Maryland, USA). Finally, the average value of SGN density was expressed in cells/μm^2 from every three sections.

SGNs culture and treatment

SGNs were prepared from embryonic day-18 rats as described by Bastianetto [20]. For the dissociation of SGNs, rats were deeply anesthetized and then decapitated. The whole Rosenthal's canal was isolated and dissociated in a trypsin solution (1.25 mg/mL in Hank's Buffer salt solution) for 10 min at 37°C. The tissue was eluted with the plating medium DMEM supplemented with 10% fetal bovine serum. After three washes with culture medium, the ganglia were mechanically dissociated by trituration with a mechanical pipettor with 1 ml pipette tips. After centrifuged and re-suspended, the SGN were plated at a density of 1×10^5 cells/ml in 10 g/ml poly-L-lysine coated 24 well or 96 well culture plates in a humid atmosphere of 5% CO_2. The SCNs were pretreated with vincamine (20-80 μM) for 1 h, and then treated with 2 mM glutamate for 23 h.

SGNs viability assays

To better understand the effects of vincamine on cell viability, MTT method was performed.

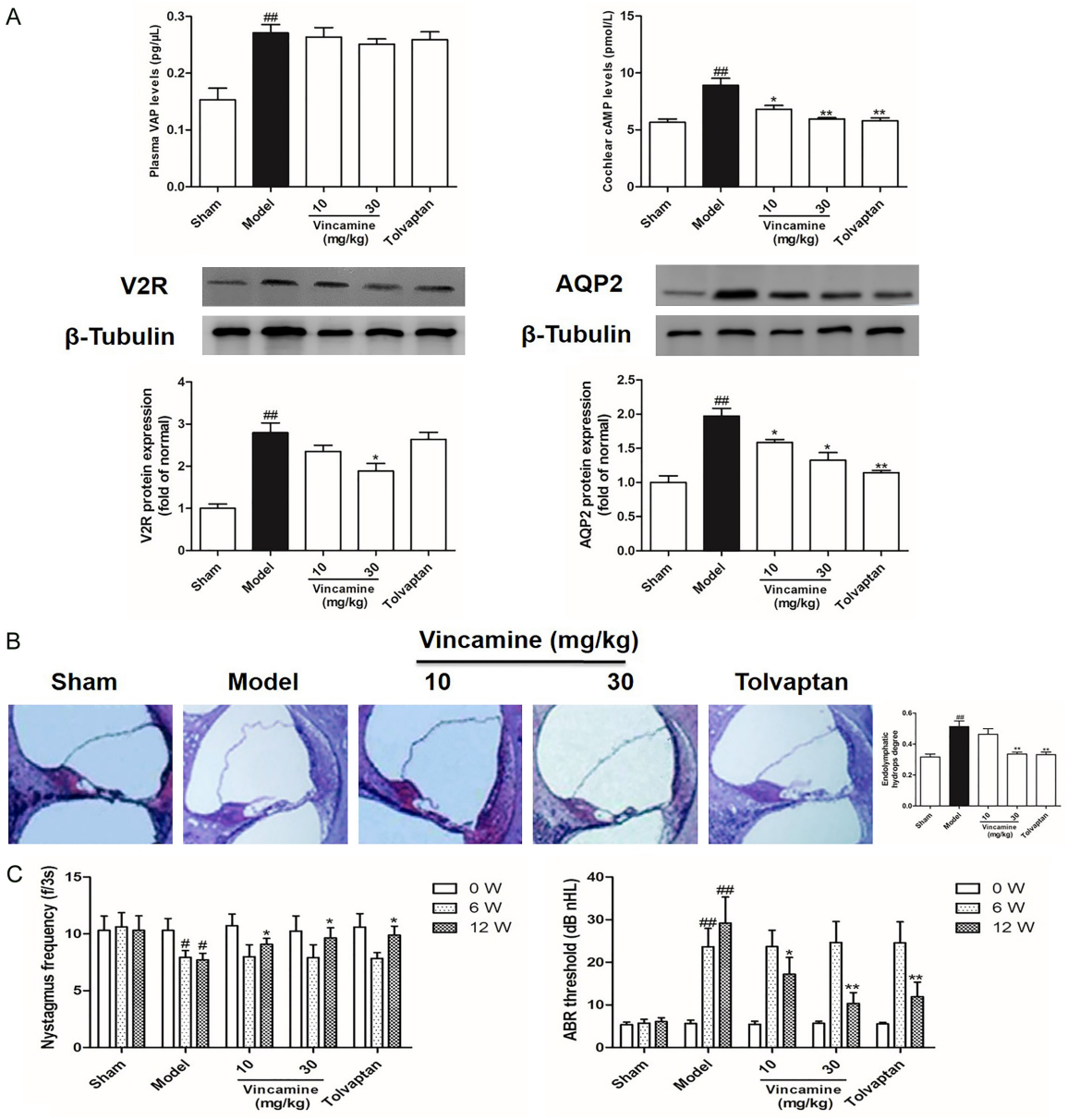

Figure 2. Vincamine down-regulated the cAMP, V2R, AQP2 levels, reduced the cochlear hydrops degree, improved spontaneous nystagmus and auditory brainstem response in ELH guinea pigs. ELH guinea pigs were treated with saline and/or vincamine (10 or 30 mg/kg/d) and/or tolvaptan (10 mg/kg/d) for 6 weeks. A. Plasma-AVP, cochlea-cAMP contents were measured using commercial assay kits, while cochlea-V2R, cochlea-AQP2 expression levels were analyzed via western blot with β-Tubulin as an invariant control for equal loading. Representative blots are shown with densitometry. Original full-size blots are presented in Figure S1. B. Representative examples of right cochlea from each experimental condition after treatment. Histological analysis confirmed the changes in endolymphatic space. The hydrops quantification was counted according to the ratio of SM area to SM + SV area (HE staining). C. Spontaneous nystagmus and auditory brainstem response were tested before and 6, 12 weeks after the surgery. Data are expressed as mean ± S.D. #P < 0.05, ##P < 0.01 vs sham; ***P* < 0.01, **P* < 0.05 *vs.* model.

SGNs were seeded in 96 well plates and incubated at 37°C in 5% CO_2. Subsequently, MTT (5 mg/mL, 20 μL) was added into each well according to the manufacturer's instructions. The absorbance of each well was measured at 570 nm with a microplate reader (Bio-Rad, Hercules, California, USA).

SGNs apoptosis and necrosis observations

To observe the apoptotic changes in SGNs, the apoptosis/necrosis dual-staining was conducted as described by Zhou [21]. After treated by vincamine, SGNs were washed twice by PBS, fixed with 95% alcohol for 10 min, and then

stained by Ho.33342 (10 mg/mL) and PI (50 mg/mL) at 37°C for 30 min. Thereafter, SGNs were visualized under a fluorescence microscope (Nikon TE2000-U, Shizvoka, Japan).

Spectrophotometer detections and enzyme-linked immunosorbent assays (ELISA)

After treated with different concentrations of vincamine, the plasmic AVP, cochlear cAMP, cochlear MDA, cochlear SOD, cochlear GSH-Px, intracellular MDA, intracellular SOD, intracellular GSH-Px, intracellular NGF, intracellular BDNF, intracellular NT3 and intracellular NT4 levels were measured using commercial assay kits (Jiancheng, Nanjing, China) according to the manufacturer's protocols.

Immunofluorescent staining

SGNs were grown on glass coverslips. After treatment for the indicated time periods, the cells were fixed with 4% paraformaldehyde for 20 min, and then permeabilized with 1% Triton X-100 for 10 min. After further washing, the cells were blocked with 10% goat serum for 30 min at room temperature. Then the cells were incubated with anti-Trks (1:250, Abcam, Cambridge, USA) antibody at room temperature for 2 h, followed by the incubation of secondary antibody for 1 h. The nuclear was counterstained with Hoechst 33342 solution (5 mg/ml in PBS) for 10 min. The coverslips were mounted on glass slides with anti-fade mounting media (Invitrogen, USA), accompanied with immunofluorescent visualization using a fluorescence microscope (Nikon, Tokyo, Japan). Densitometric analysis was performed using Image-Pro Plus 6.0 software. Results were from triplicate experiments.

Western blot analysis

Total proteins were extracted from ELH-cochleae and SGNs by applying RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% SDS, 1% Triton X-100, 2 mM EDTA). After 30 min, protein concentrations were determined using BCA Quantification Kit (Beyotime, Beijing, China). Each protein sample was separated using 10% SDS-PAGE, and then transferred onto PVDF membrane (Millipore, Darm stadt, Hesse, Germany). The PVDF membrane was blocked with 5% BSA (w/v) at room temperature for 1 h, and then incubated with primary antibodies (1: 2000, Cell Signaling Technology, Beverly, Massachussets, USA) at 4°C for the whole night. Thereafter, the PBST was used to wash the membrane thrice, then the membrane was incubated with secondary antibody (Boster Biotechnology, Wuhan, China) for 2 h. The immune reactivity was visualized using the ECL Western blotting kit (Theromfisher, Waltham, Massachusetts, USA) according to the manufacturer's protocols.

Statistical analysis

All data were presented as mean ± standard error on the mean (SEM). Statistical differences between two groups were analyzed using unpaired Student's t tests. Differences among multiple groups were analyzed using one-way ANOVA. $P < 0.05$ was considered indicative of statistically significant differences.

Results

Vincamine inhibited the VAP/AQP2 axis function in ELH-related experimental models

The relative experimental procedures were performed to detect the VAP concentration in plasma, and the V2R, cAMP and AQP2 (aquaporin2) levels in right cochlea (operated ear) after the treatment of vincamine. As shown in **Figure 2A**, VAP, V2R, cAMP and AQP2 levels were elevated in ELH-models. In contrast, vincamine at different concentrations (10, 30 mg/kg) showed prominent reduction on the V2R, cAMP and AQP2 levels (**Figure 2A**). However, the vincamine had no effect on plasma-VAP, possibly because AVP was released from posterior pituitary and might be free from the regulation of vincamine. Based on the above results, we speculated that vincamine might reduce the ELH by down-regulating the downstream components of VAP/AQP2 system. To further verify the hypothesis, we examined the pathological changes in cochlea by HE staining method.

Vincamine reduced cochlear hydrops degree in ELH guinea pigs

Preliminarily, distinct endolymphatic hydrops was observed in model group, and the distension of Reissner's membrane was also obvious in comparison to sham group. However, vincamine could alleviate the pathological alterations in a dose dependent manner (**Figure 2B**). To objectively quantify the degree of endolym-

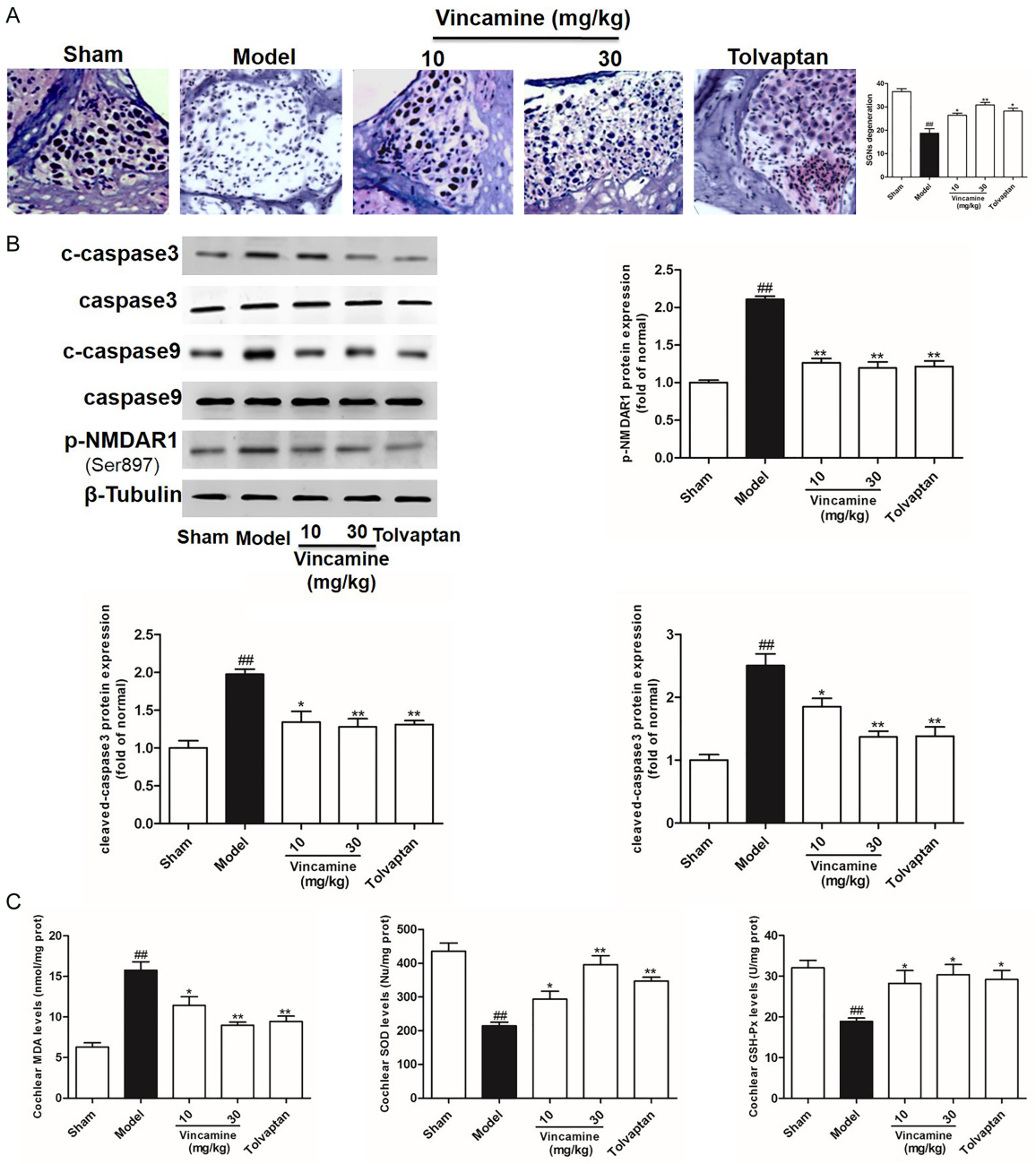

Figure 3. Vincamine inhibited the degeneration of SGNs, eliminated the accumulation of p-NMDAR1, c-caspase3/9 and MDA, and induced the secretion of SOD, GSH-Px in ight cochlea of ELH guinea pigs. ELH guinea pigs were treated with saline and/or vincamine (10 or 30 mg/kg/d) and/or tolvaptan (10 mg/kg/d) for 6 weeks. A. Observation and quantification of spiral ganglion neurons density in right cochlea. B. Related protein extractions of cochlear tissue were analyzed via western blot with β-Tubulin as an invariant control for equal loading. Representative blots are shown with densitometry. Original full-size blots are presented in Figure S2. C. MDA, SOD and GSH-Px contents were measured using commercial assay kits. Data are expressed as mean ± S.D. ##P < 0.01 vs sham; ***P* < 0.01, **P* < 0.05 *vs.* model.

phatic hydrops, the ratio of SM area to SM + SV area was calculated in the cochlear duct areas [22]. The results showed that vincamine could significantly decrease the ratio values in ELH guinea pigs (**Figure 2B**). All these *in vivo* data ultimately confirmed the therapeutical effects of vincamine on ELH via VAP/AQP2 signaling pathway, which suggested that vincamine might be a potential agent for developing novel therapies in ELH-associated MD.

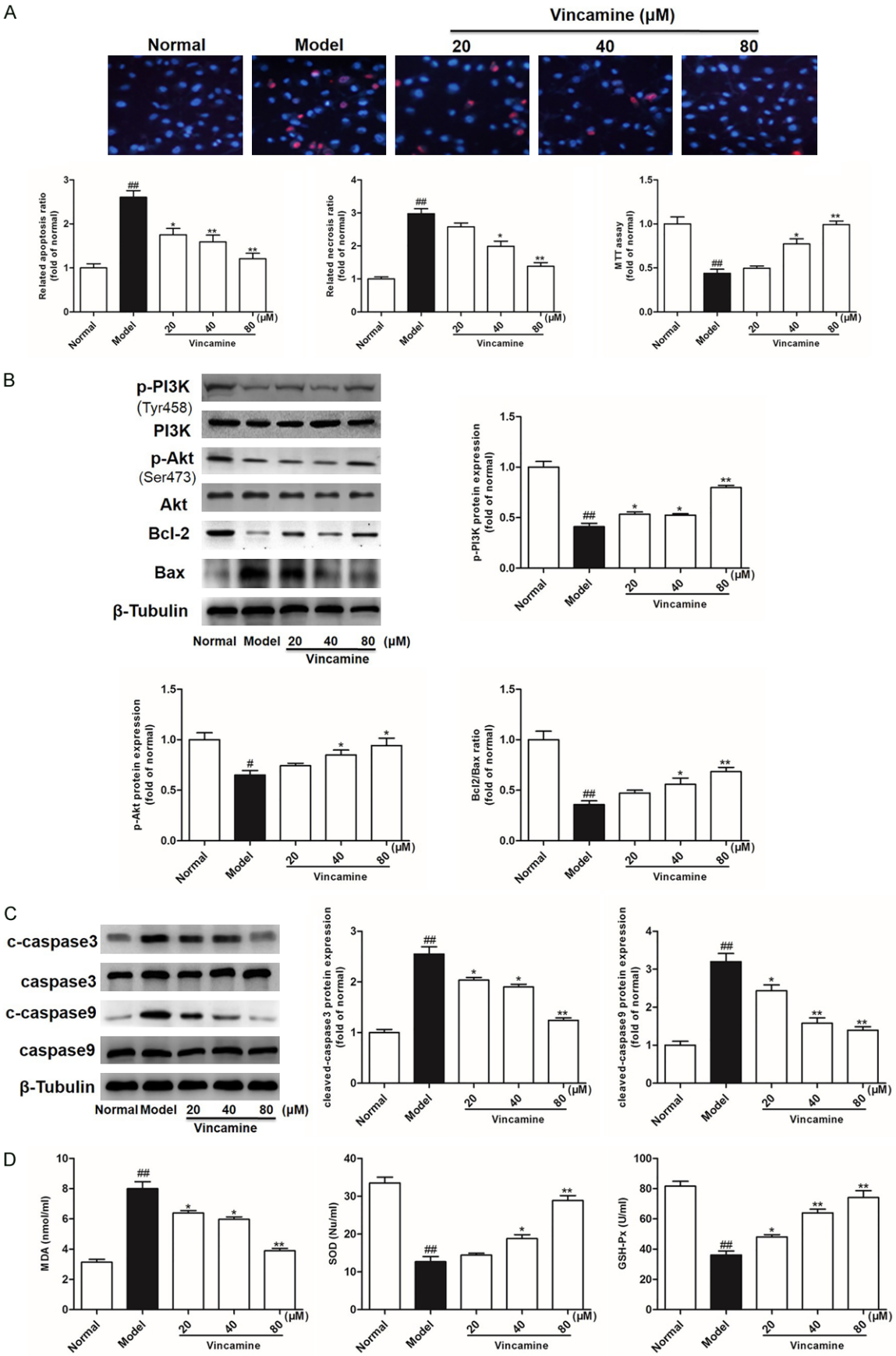
A
Vincamine (μM)
Normal
Model
20
40
80
Related apoptosis ratio (fold of normal)
Related necrosis ratio (fold of normal)
MTT assay (fold of normal)
Normal
Model
Vincamine
(μM)
B
p-PI3K (Tyr458)
PI3K
p-Akt (Ser473)
Akt
Bcl-2
Bax
β-Tubulin
Normal Model 20 40 80 (μM)
Vincamine
p-PI3K protein expression (fold of normal)
p-Akt protein expression (fold of normal)
Bcl2/Bax ratio (fold of normal)
C
c-caspase3
caspase3
c-caspase9
caspase9
β-Tubulin
Normal Model 20 40 80 (μM)
Vincamine
cleaved-caspase3 protein expression (fold of normal)
cleaved-caspase9 protein expression (fold of normal)
D
MDA (nmol/ml)
SOD (Nu/ml)
GSH-Px (U/ml)

Figure 4. Vincamine prevented cell apoptosis and necrosis, improved cell survival, up-regulated p-PI3K, p-Akt and Bcl-2/Bax levels, eliminated c-caspase3/9 and MDA accumulation, and induced SOD, GSH-Px secretion in glutamate-injuried SGNs. SGNs isolated from embryonic rats were pretreated with vincamine (20-80 μM) for 1 h, and then treated with 2 mM glutamate for 23 h. A. SGNs apoptosis, necrosis and viability were measured using hoechst/PI dual-staining and MTT methods, respectively. B, C. Cell lysates were subjected to western blotting test and probed for the expression levels of PI3K, p-PI3K, Akt, p-Akt, caspase-3, cleaved-caspase3, caspase-9, cleaved-caspase9, Bcl-2 and Bax and β-Tubulin as the loading control. Representative blots are shown with densitometry. Original full-size blots are presented in Figure S3. D. MDA, SOD and CSH-Px levels in SGNs were tested according to the commercial assay kits. Data are expressed as mean ± S.D. #P < 0.05, ##P < 0.01 vs normal; ***P* < 0.01, **P* < 0.05 *vs.* model.

Vincamine protected the vestibular function and hearing in ELH-related experimental models

Before ELH surgery, no apparent vestibular dysfunction was identified from the daily behavior of the guinea pigs, and there were no significant differences among the animals in ENG and/or ABR tests. However, differences were found after surgery for 6 weeks. The operated animals suffered from characteristic head tilt, postural disturbances and hearing disorder, accompanied with nystagmus frequency decreased and ABR threshold increased. But there were no obvious changes in sham-opreated animals. Immediately after the treatment with vincamine for another 6 weeks, hearing loss and spontaneous nystagmus were markly recovered (**Figure 2C**). Therefore, it is hypothesized that the vertigo attack and hearing losss might be alleviated if the ELH could be eliminated by vincamine.

Vincamine relieved SGNs degeneration by mediating the glutamate excitotoxicity in cochleae

In Meniere's disease, SGNs degeneration are usually regarded as the mainly cause of ELH-related hearing loss [23]. The results of SGNs density detection supported the previous studies that ELH could aggravate SGNs degeneration in guinea pigs. However, SGNs density was markedly increased after the vincamine treatment (**Figure 3A**). Glutamate is generally treated as the inducement in many neurological disorders, and is also a potential pathogenic factor in ELH-associated SGNs degeneration [24]. To clarify whether the activity of glutamate involved in the SGNs degeneration, phospho-NMDAR1, cleaved-caspase-3 and cleaved-caspase-9 expression levels were measured using WB analysis. Concurrently, commercially available kits were used to detect the MDA, SOD and GSH-Px concentrations in cochlea. Our results showed that vincamine significantly decreased the phospho-NMDAR1, cleaved-caspase-3 and cleaved-caspase-9 expressions in a dose-dependent manner (**Figure 3B**). Vincamine could also reduce the tissue levels of MDA, SOD and GSH-Px (**Figure 3C**). Notably, it concluded that the anabatic effects of glutamate on ELH-associated SGNs degeneration might be through the up-regulation of phospho-NMDAR1, which further activated the oxidative stress to induce the SGNs damage. Nevertheless, vincamine protected the SGNs by resisting the glutamate-associated undesirable effect in cochleae.

Vincamine promoted the viability, inhibited the apoptosis and necrosis of glutamate-injured SGNs

To further ensure the protective effect of vincamine, Hoechst/PI dual staining of SGNs was performed to detect the cell apoptosis and necrosis, and then MTT method was carried out to measure the cell viability. In the visual fields, necrotic cell nuclei was stained with red fluorescence, and apoptotic cell nuclei was stained with brilliant-blue fluorescence. As a result, the glutamate markedly increased the necrotic and apoptotic percentages in SGNs, whereas vincamine distinctly alleviated the changes (**Figure 4A**). Next, MTT test in **Figure 4A** indicated that glutamate reduced the SGNs viability visibly, which could be improved by vincamine in a dose-dependent manner. So we came to the conclusion that vincamine could suppress the cell apoptosis and necrosis, promote the cell viability of glutamate-injured SGNs.

Vincamine regulated PI3K/Akt signaling pathway related molecules

As shown in **Figure 4**, WB analysis was carried out to elucidate the mechanism of vincamine in relieving ELH-associated SGNs degeneration. Representative blots showed the expression

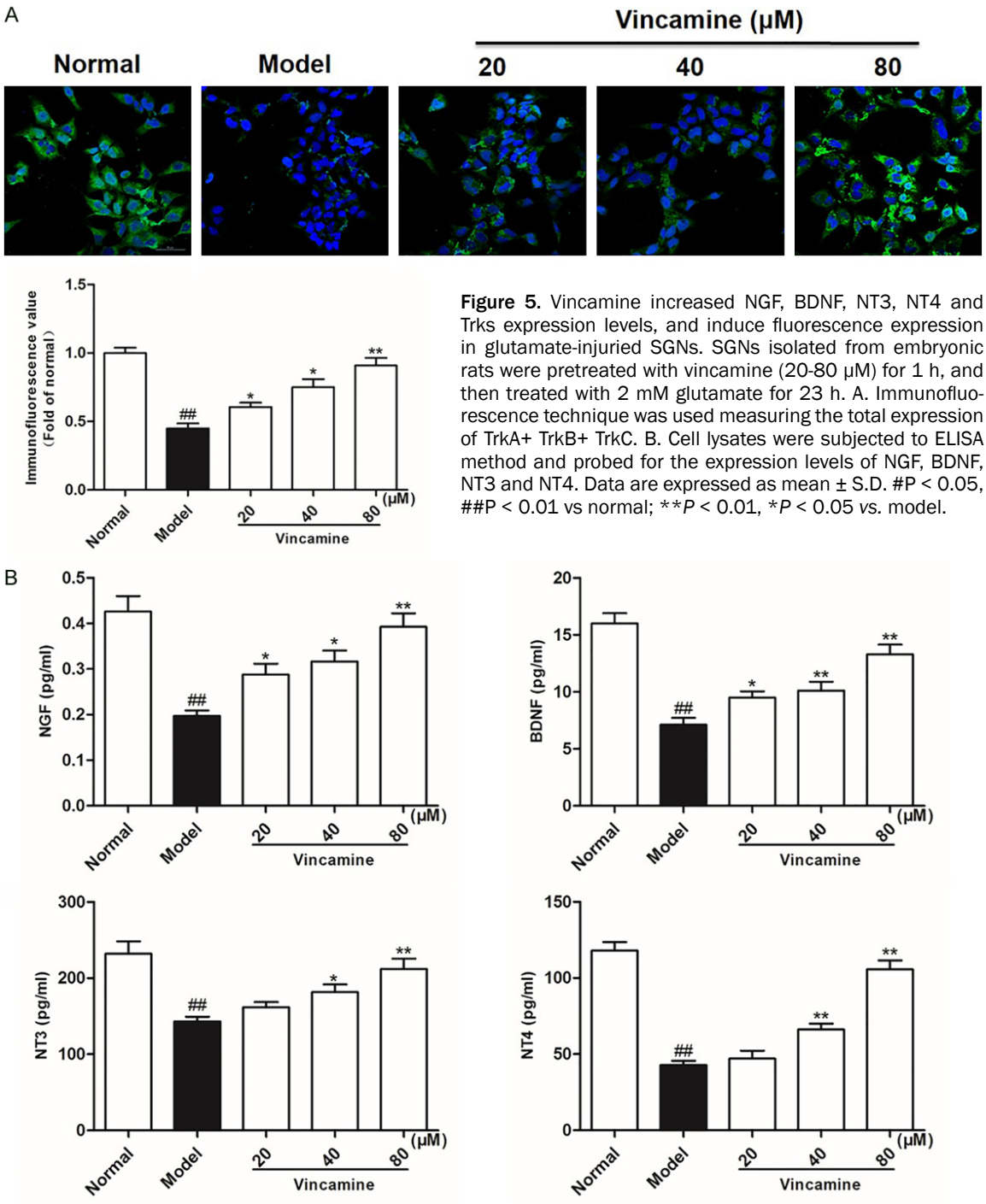

Figure 5. Vincamine increased NGF, BDNF, NT3, NT4 and Trks expression levels, and induce fluorescence expression in glutamate-injuried SGNs. SGNs isolated from embryonic rats were pretreated with vincamine (20-80 μM) for 1 h, and then treated with 2 mM glutamate for 23 h. A. Immunofluorescence technique was used measuring the total expression of TrkA+ TrkB+ TrkC. B. Cell lysates were subjected to ELISA method and probed for the expression levels of NGF, BDNF, NT3 and NT4. Data are expressed as mean ± S.D. #P < 0.05, ##P < 0.01 vs normal; ***P* < 0.01, **P* < 0.05 *vs.* model.

levels of proteins involved in the PI3K/Akt signaling pathway. The relative data demonstrated that glutamate signifcantly reduced phosphorylation levels of PI3K and Akt. It also reduced the expression of Bcl-2, and increased the expression of Bax (**Figure 4B**). The cell damage biomarkers such as cleaved-caspase3 and cleaved-caspase9 were also increased in glutamate-treating SGNs (**Figure 4C**). However, pretreatment with vincamine could instantaneously alleviate the glutamate-induced changes in a dose-dependent manner. Previous study has disclosed that oxidative stress was important in inducing SGNs injury by glutamate [25]. The results in our research showed that vincamine could decrease the MDA level, and increase SOD and GSH-Px levels, which declared the antioxidative abilities of vincamine in gluta-

mate-injured SGNs (**Figure 4D**). Nevertheless, pretreatment with vincamine could promote the NGF, BDNF, NT3, NT4 and Trks expression levels in glutamate-injuried SGNs (**Figure 5A** and **5B**). Therefore, all these *in vitro* data further confirmed the protection of vincamine on SGNs, suggested that therapeutical effect of vincamine on SGNs against glutamate-induced damage was militated via the neurotrophin-dependent PI3K/Akt signaling pathway.

Discussion

Meniere's disease is a heterogeneous syndrome accompanied with numerous etiologies, such as genetics, autoimmunity migraine, inflammatory reaction, anatomical variations in the temporal bone, and altered intra labyrinthine fluid dynamics [26-28]. Careful temporal bone studies have confirmed that all patients diagnosed with MD exhibited ELH in at least one ear [29]. Therefore, experimentally models of endolymphatic hydrops, which created by surgical ablation of the endolymphatic duct and sac in guinea pigs, were widely used to investigate the pathological changes of MD associated with ELH [30, 31]. Certain findings have provided the evidence that abnormal microcirculation may underlie the pathology of ELH-associated MD [32]. Clinical and experimental studies have also revealed that AVP/AQP2 pathway played an important role in maintaining microcirculation, which caused the occurrence of ELH in MD [33]. Previous studies have principally reported that the V2R was distributed in the renal collecting duct, and the activation of V2R could increase the levels of cAMP. Afterwards, the accumulation of cAMP would stimulate the expression of AQP2, which further accelerate the water reabsorption in renal [34]. Currently, it has speculated that the AVP/AQP2 pathway also played a crucial role in the inner ear [35]. In MD patients, the AVP levels are increased in the circulation [36]. Excessive AVP promotes the cAMP activity in endolymphatic sac of Meniere's patients, and then the AQP2 level in the cochlea is specifically up-regulated under cAMP stimulation [37, 38]. Accordingly, the increased AVP level expedites the production of endolymph in hydropic cochlea via AVP/AQP2 pathway. Tolvaptan is a selective, competitive vasopressin receptor 2 antagonist, has been proven to be an effective option in ELH by regulating AVP/AQP2 system in the inner. Therefore, we regarded tolvaptan as the positive control in the following experiments [39]. Combined with the previous researches, the AVP/AQP2 signaling pathway was detected. Based on the related experiments, we found that vincamine could significantly suppress the cAMP, V2R and AQP2 levels in cochlea. Observation from HE staining showed the protective effects of vincamine on the cochlear pathological changes. ABR and ENG tests also disclosed the repair effects of vincamine on auditory and vestibular disfunctions induced by ELH. Notably, these results indicated that vincamine suppressed the formation of ELH in MD by inhibiting the AVP/AQP2 signaling pathway, which might potentially benefit the ELH associated MD patients. Moreover, our results also revealed a tendency for the SGNs degeneration in association with ELH, was significantly alleviated by vincamine. It demonstrated the SGNs-protective effect of vincamine in ELH, and the pharmacological mechanism should be further excavated.

In Meniere's disease, studies suggest that principle pathologic signs of ELH related hearing loss are primary due to SGNs degeneration [23]. Glutamate plays a critical role in ELH-related MD, which acts as a neurotransmitter involved in signal transduction between the inner hair cells and SGNs interfaces, aggravates the cochlear damage under ELH condition [40]. Generally, excitotoxic mechanisms of glutamate in common neurological disorders are analogous to that in ELH-associated hearing loss [24]. The glutamate-associated undesirable effect may be the dependable treating target in improving the ELH-associated SGNs damage, and preventing or remiting the ELH-induced hearing loss. Notably, the bind between glutamate and postsynaptic N-methyl-D-aspartate receptor (NMDAR) plays an important role in maintaining the SGNs vitality. Phosphorylation of NMDAR1 (Ser897), is known to enhance the NMDAR activity primarily. But the superfluous glutamate stimulates the NMDAR unduly, which causes the intracellular calcium overload and further activates calcium related signaling pathways. Finally, the oxygen free radicals production and cell damage are induced [41, 42]. Glutamate-induced oxidative toxicity is characterized by various detrimental changes in GSH-Px, MDA and SOD levels, which means that oxidative stress plays an important role in gluta-

mate-induced SGNs injury [25]. The oxidative stress increases both permeability and function of mitochondrion, which results in cell apoptosis [43]. In apoptotic cells, caspase-3 acts as the effector caspase, is activated by caspase-9. Generally, caspase-3 and -9 are usually measured to ascertain the cell apoptosis [44]. Therefore, whether neuroprotective agents, such as vincamine, can protect ELH-induced SGNs damage by mediating the glutamate excitotoxicity is a meaningful question to be solved. Supporting the hypothesis, the current study demonstrated that vincamine remarkably decreased the activation levels of NMDAR1, caspase-3 and caspase-9 in cochlea, reduced the cochlear MDA concentration, and enhanced the GSH-Px and SOD levels in cochlea. Taken together, such novel results strongly suggested that vincamine was able to protect SGNs against ELH-associated degeneration, and the intervention of glutamate-induced damage might partially contributed to the protective effects of vincamine.

In the recent years, researchers pointed out that numerous signaling pathways participating in the neuroprotective effects of reagents, which were used to treat the glutamate-induced excitotoxicity. Among them, the PI3K/Akt signaling pathway has received considerable attention for its crucial role in protecting neuronal cells against glutamate-associated degeneration [45, 46]. Previous studies have also indicated that PI3K/Akt pathway played an important role in protecting cells from caspase mediated apoptosis [47, 48]. As mentioned above, caspase-3 and caspase-9 are the critical downstream factors involved in glutamate-induced apoptosis, are also identified as the downstream targets of PI3K/Akt pathway [49]. In many systems, the decrease in Bcl-2/Bax ratio causes the apoptosis, and induces the release of caspase-3/-9. Members of the Bcl-2 family modulate apoptosis, with the Bcl-2/Bax ratio serving to as a rheostat to determine cell susceptibility to apoptosis [50]. Since PI3K/Akt pathway plays a key role in the apoptotic mediation of Bcl-2 and Bax [51]. Activation of PI3K may promote neuronal survival through regulation of Bcl-2 family proteins, by activating Akt kinase. In our study, vincamine enchanced the viability of glutamate-injured SGNs, and then suppressed the cell apoptosis and necrosis. The increased levels of p-PI3K, p-Akt and Bcl-2, accompanied with decreased levels of Bax, cleaved-caspase3 and cleaved-caspase9 levels were also found in vincamine-treated SGNs. Moreover, the PI3K/Akt pathway has been implicated in modulating oxidative stress levels [52]. In the current study, the levels of SOD, GSH and MDA were rapidly recovered by vincamine. Thereafter, we speculate that vincamine may activate the Bcl-2/Bax signaling by mediating the PI3K/Akt signaling pathway, and finally alleviate the glutamate-induced excitotoxicity in SGNs. It is well known that neurotrophins are a family of proteins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin3 (NT3), and neurotrophin4 (NT4) [53]. Furthermore, tropomyosin-related kinases (Trks) are the common receptors of neurotrophins, which play crucial role in neurotrophin mediated cellular activities, such as neuronal differentiation, survival, and synaptic function [54], via PI3K/Akt signaling pathway [55]. Our present study showed that vincamine could significantly induce the synthesis of NGF, BDNF, NT3 and NT4, and increase the fluorescence expression of Trks. Taken together, these results indicated that vincamine could protect SGNs against glutamate-induced excitotoxicity, and its neuro-protection might be at least modulated by neurotrophin-dependent PI3K/Akt signaling pathway.

As a conclusion, the present study is the first to show the potential value of vincamine in treating ELH-associated MD. Our results suggest that the vincamine suppress the formation of ELH in MD is through the AVP/AQP2 pathway. The data also indicate that neurotrophin-dependent PI3K/Akt signaling pathway is a critical target of the vincamine mediated protection in ELH-related SGNs injury. Taken together, our novel findings will be valuable in understanding the effective actions of vincamine, with great potential for alleviating the endolymphatic hydrops related vertigo attack and hearing loss in Meniere's disease.

Disclosure of conflict of interest

None.

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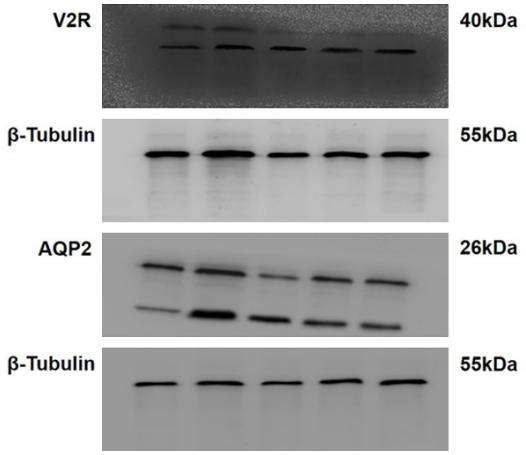

Figure S1. Vincamine inhibited the expression levels of cochlea-V2R and cochlea-AQP2. Related protein extractions of cochlear tissue were analyzed via western blot.

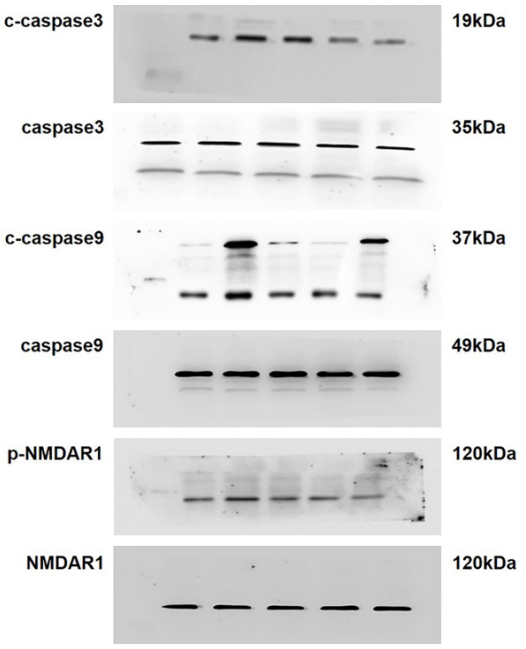

Figure S2. Vincamine eliminated the accumulation of c-caspase3, c-caspase9 and p-NMDAR1. Histiocyte lysates were subjected to western blotting test and probed for the expression levels of cleaved-caspase3, caspase3, cleaved-caspase9, caspase9, p-NMDAR1 and NMDAR1.

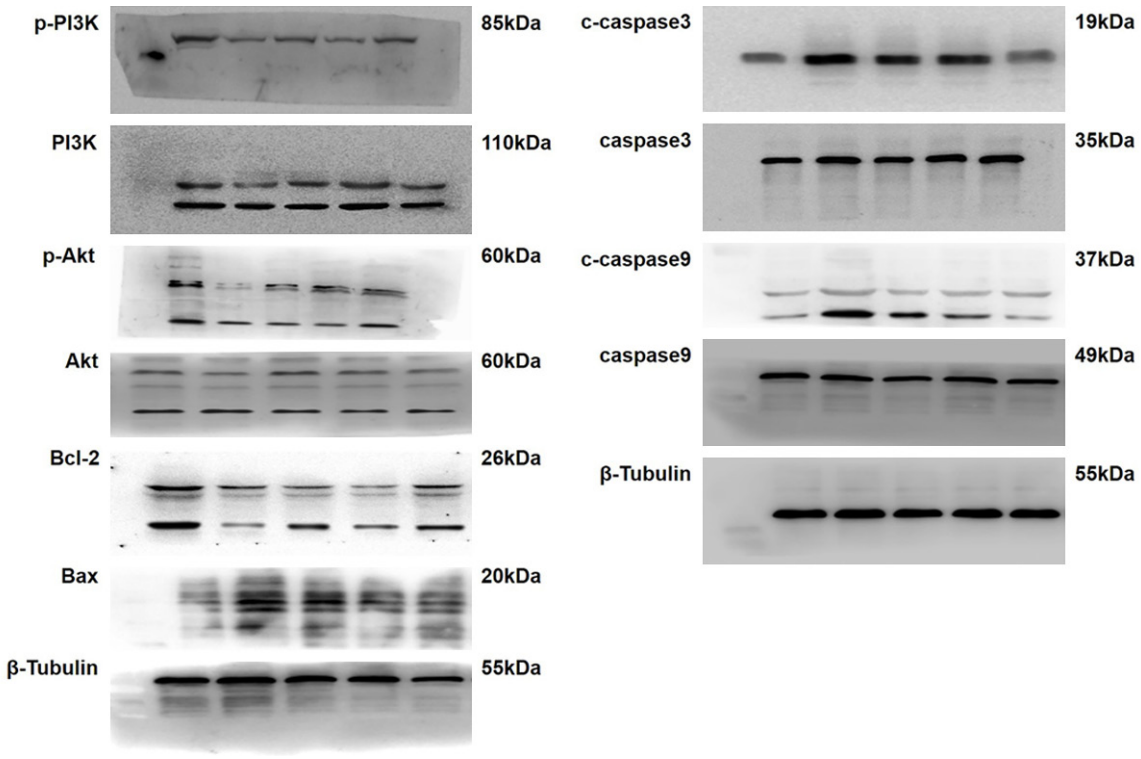

Figure S3. Vincamine up-regulated p-PI3K, p-Akt and Bcl-2/Bax levels, eliminated c-caspase3 and c-caspase9 accumulation. Cell lysates were subjected to western blotting test and probed for the expression levels of p-PI3K, PI3K, p-Akt, Akt, Bcl-2, Bax, cleaved-caspase3, caspase3, cleaved-caspase9 and caspase9.