Protection Induced By Ginsenoside Rb1 in Alzheimer’s Disease Model is Associated With Redressing The Neuronal Hyperexcitability Which is Regulated By Nav1.2 and Nav1.6

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**Abstract**

**Background:** Alzheimer’s disease (AD), a neurodegenerative disease showing multiple complex pathomechanism alterations, has affected the normal life of many old people. It is urgent to find an effective medicine for AD. Ginsenoside Rb1 (Rb1) is the main component of panax notoginseng saponins, a famous Chinese herbal medicine, and is expected to be a useful drug in the treatment of AD. This study mainly explored the potential effects of Rb1 in model of AD in vivo and in vitro, and investigated the possible mechanisms.

**Materials and methods:** We studied the neuroprotective effect of Rb1 in transgenic mouse animal model and cell AD model in vitro. The cognitive function of APP/PS1 mice was measured in Morris water maze and Novel Object Recognition. Electrophysiological patch clamp recording and electrophysiology were used to nerve excitability. Further, the expression of proteins of Nav1.2 and Nav1.6 were used by Western blot.

**Results:** We found that Rb1 treatment significantly improved the cognitive and memory loss, and reversed the hyperexcitability by altering the expressions of Nav1.2 and Nav1.6 of APP/PS1 mice. Further, Rb1 improved the hyperexcitability induced by Aβ1-42-injured neurons, which might be associated with alteration of the expressions of Nav1.2 and Nav1.6. As we expect, Rb1 attenuated Aβ1-42-induced injury in primary neurons.

**Conclusion:** Our data showed that Rb1 played a critical role in improvement of the cognitive deficit and abnormal excitability of AD by regulating Nav1.2 and Nav1.6 expressions. Thus, Rb1 shows protective effects on AD models and may be a potential candidate for AD treatment.

**Introduction**

Alzheimer’s disease (AD) is the main reason of dementia in elderly individuals, which is characterized by progressive memory loss and cognitive impairment[1, 2]. AD is a widespread disease that has affected more than 50 million people, and there is currently no effective cure for it [3]. Thus, it is urgent to find an effective medicine for AD.

Panax notoginseng (PNS) is a prominent traditional Chinese herb composed with multiple pharmacological activities and is proved to be effective for various diseases treatment, including cardiovascular disease, cancer and other diseases [4]. Recent reports showed that PNS have neuroprotective effects in global cerebral ischemia–reperfusion (I/R) and spinal cord I/R injuries [5]. Meanwhile, PNS is a medicine made of two or more ingredients, of which ginsenoside Rb1(Rb1) is a major component[6]. Rb1 has been shown to enhance the neuroprotective effect of neurons by upregulating brain-derived neurotrophic factor (BDNF) [7, 8]. In addition, Rb1 was reported to improve spatial learning and memory by regulating hippocampal neurogenesis, hippocampal synaptic density and α-synuclein/PSD-95 pathway [9-11]. Interestingly, ginsenoside R1 is thought to improve memory...
function and synaptic plasticity in ways that improve neuroexcitability[12]. However, whether Rb1 have similar pharmacological functions as R1 has not been clarified.

Voltage-gated sodium channels (Nav) play key roles in excitable cells that initiate and propagate action potential[13]. There are nine mammalian Nav channel isoforms (Nav1.1-Nav1.9), which are important in excitatory cells such as nerve cells[14]. Nav1.1 was showed to have ability to alter the cognitive or behavioral impairment by impacting GABAergic neurons[15]. Furthermore, the activation of Nav1.1 channels have anti-convulsive effects[16], while the Nav1.2 and Nav1.6 are firstly found to be expressed on the unmyelinated fibers and axon initial segments, respectively[17]. Recent study also revealed the increase of Nav1.1, Nav1.2 and Nav1.6 current stimulated the microglial activation and induced inflammation following spinal cord injury [18]. In addition, either Nav1.2 or Nav1.6 plays crucial roles in formation of seizures [19]. It has been evidenced that Nav1.2 affects susceptibility and behaviour of seizure by decreasing the neuronal excitability[20]. The abnormal expression and distribution of Nav1.6 was reported to be associated with various neurological disorders that characterized by neuronal hyperexcitability[21]. Moreover, we found that exercise training improved cognitive deficient of APP/PS1 mice mainly by altering the expression and distribution of Nav1.1α, Nav1.2 and Nav1.6[22]. Therefore, to maintain the normal expressions and functions of Nav1.1α, Nav1.2 and Nav1.6 may be the key target for various treatments induced-improvement in cognitive and memory of AD.

Taken together, this study aimed to explore the effects of Rb1 in cognitive and memory impairment of AD mice, and whether the potential mechanism is associated with neuronal excitability.

**Materials And Methods**

**Preparation of Ginsenoside Rb1**

Ginsenoside Rb1 (C_{54}H_{92}O_{23}, molecular weight 1109.29, purity >98.0%) was purchased from the MedChemExpress LLC (cas no. 41753-43-9, Shanghai, China). Rb1 was dissolved in dimethyl sulphoxide (DMSO) to make a 1 mM reserve solution, which was deposit in -20˚C.

**Animals**

APP/PS1 double transgenic mice and wild-type (WT) mice, aged 2 months, were obtained from laboratory animal center of Kunming medical university (Kunming, China). The animals were housed under a room a temperature of 22± 2 °C with light control based on a 12h light-dark cycle. All mice were supplied with unlimited food and water. All experiments were approved and conducted in compliance Guidelines of the Institutional Animal Care Committee of Kunming University, China (license number: kmu-eac-2018021; Kunming, China).

**Experimental design**
The APP/PS1 mice were randomly divided to the two groups. One group was treated with ginsenoside Rb1 (APP/PS1+Rb1 group), and another group was treated with 10% sterilized DMSO in place of Rb1 (APP/PS1+vehicle group). Each mouse in the APP/PS1+Rb1 group was administered with 5 mg/kg Rb1 once a day for 6 months by gavage. After treatment, the Morris water maze (MWM), the novel object recognition (NOR) task and electroencephalogram (EEG) recordings were performed to detect the potential alterations induced by Rb1 treatment in APP/PS1 mice. One week post the EEG recording, all mice were sacrificed to obtain the cortex and hippocampus for subsequent analyze.

NOR task

The NOR task is last for three days and all mice were placed in a transparent plastic box with size of 40×40×40 cm³. The first day was used as a habituation phase, while the second day was used as exploratory phase and last day was used for testing. During the habituation phase, each mouse were administration in the apparatus without objects in 5 minutes. 24 h later, two identical objects were placed into the box and mice had the opportunity to explore for 5min. On the last day, one of the old objects was substitute for a novel object (NT) in the apparatus. Each mouse has the right to explore novel object (FT) and familiar object (FT) freely, subsequently the time spend was recorded. The cognitive function of mice was evaluated by the discriminant index (NT-FT)/(NT+FT).

MWM tests

The MWM test was carried out as previously described [23]. Mice were trained individually in a circular pool (diameter, 100 cm; height, 50 cm) filled with water (24±1°C, 20 cm depth). The day before the regular testing, all mice were habituated to the water and allowed them step on a white platform (hidden below the water surface) to rescued themselves. Briefly, a mouse was subjected to the swim around for 10 seconds(s) and afterwards allowed to stay the white platform submerged under water for only 1 to 2 s. During the routine testing, the escape platform was placed in different position. The time that elapsed until the mouse reached the escape platform was noted as escape latency, which lasts 5 days. On the 6th day, the escape platform was moved from tank and the probe testing was performed. The mouse swam for 60s to find the platform location.

The time spent by each mouse in the target quadrant, and the numbers that they entered the quadrant were recorded. A computer tracking program was used to track the animal path, and an animal behavior video analysis system (Xinruan Information Technology Co., Ltd., Shanghai, China) was applied to analyzed the data.

EEG recordings

For EEG experiments, recording was conducted in each mouse using the polyimide-based microelectrode array (PBM-array). The protocols in this research used a method published in some literature previously[24].
The mouse was anesthetized with isoflurane (2–3% for induction) mixed with O₂ (0.5-1 L/min) for electrode fixation before EEG recordings. The mice were then implanted into the hippocampus (AP-2.1mm, ML-1.5, DV-1.5mm) with three bare electrodes with exposed tips of polyimide coating. Two miniscrews were fixed on the occipital bone above the cerebellum, which were used as ground and reference respectively. Connect the connector to the multi-channel EEG amplification system to collect neuroelectric signals. The CED Micro1401 data acquisition system digitizes the analog signal with a sampling frequency of 1khz. Signal processing ensured that in the epoch every 30 seconds, the integer value showed the dominant frequency (DF) in Hz. The high frequency time is calculated by adding the 30 s epochs numbers for DF> 6 Hz and dividing by the total number of 30 s epochs per mouse (total recording time)[25, 26].

**Western blot**

One week later, after the EEG recording, the mice were killed by cervical dislocation and decapitation. Thereafter, the brain was taken out and stored at -80 ºC for western blot and Cell-surface biotinylation assay. The levels of Nav1.1α, Nav1.2 and Nav1.6 were analyzed using Western blot. Briefly, obtained protein of cortex and hippocampus from mouse were extracted with RIPA buffer (Beyotime, Jiangsu, China). The homogenate was centrifuged at 12,000g at 4°C for 10 minutes. Then the BCA protein assay quantifies proteins. The precipitated proteins were separated on 4-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. They were then transferred to PVDF membranes and combined with Nav1.1a (1:800; cat. no. ASC-001; Alomone), Nav1.2 (1:200; cat. no. ASC-002; Alomone), Nav1.6 (1:500; cat. no. ab65166; Abcam) and GAPDH (1:800; Catalog no. Sc-365062; Santa Cruz, California, Delaware, USA). The membrane was then incubated with an appropriate secondary antibody (goat anti-rabbit IgG and goat anti-mouse IgG; ZSGB-BIO, Beijing, China, 1:5000) at room temperature for 2 hours. Finally, protein quantification was performed using enhanced chemiluminescence Luminol reagent (Beyotime, Shanghai, China). The proteins were developed by Bio-Rad Gel Imaging System (ChemiDoc™ XRS+; Bio-Rad Laboratories, Inc., Hercules, CA, USA) with Quantity One software v4.6.6 (Bio-Rad Laboratories, Inc.)

**Cell-surface biotinylation assay**

Cell surface biotinylation and NeutrAvidin pull-down was performed as previously published work[25, 26]. Briefly, the brain tissues were incubated in ice-cold Krebs solution and the preparation of solution was carried out with modification (in mM) of described previously[25]: 120 NaCl, 4.5 KCl, 1.5 KH2PO4, 10 glucose, 1.5 MgSO4, 26 NaHCO3 and 1.5 CaCl2. Biotinylated proteins were pulled down by Neutravidin-agar-agar beads (Pierce, USA). Later, the surface and intracellular levels of Nav1.1α were determined using SDS-PAGE/Western blot (as described above).

**Cell culture**

The primary cultures of cortical or hippocampus tissues from C57BL/6J mice (one-day-old) were prepared as described [27]. The prepared cortex and hippocampus tissues was incubated in 0.25% trypsin (Gibco) for 10 minutes at the 37°C and then the pancreatin was inactivated by fetal bovine serum (Gibco).
A 70-μm cell strainer used to purify the tissues and leave the neuron cells. The cells were maintained in Neurobasal medium, with 2% B27 supplement (Gibco), 0.5% penicillin-streptomycin solution and 0.25% GlutaMax (Gibco), and then seeded onto 6-well culture plates. Finally, half of culture medium was substituted with fresh portions every three days.

**Preparation of oligomer form of Aβ1-42**

The Aβ1-42 peptide was dissolved in 1, 1, 1, 3, 3, 3- Hexauoro-2-propanol (HFIP) at 1mM concentration. Afterwards, the solution was evaporated in the fume hood at room temperature for 24 h and the final clear peptide film stored at -20˚C. For oligomer preparation, films were resuspended in DMSO to 5mM, diluted at 5 mM in neural basal medium for 5h.

**Model preparation and drug deliver**

To explore the effects of Rb1 on excitability in Aβ1-42-treated neuronal cells, the Aβ1-42-treatment cultured cells were treated with 5µM Rb1 (name as Rb1 group), and the vehicle for Rb1 was DMSO (name as DMSO group). Besides, the Aβ1-42- treatment cells treated only with medium were treated as blank groups (blank group), and the baseline group is the primary neural cells untreated by Aβ1-42 and Rb1. 24h after the Rb1 treatment, the treated cells were then processed for the next detection.

**Cell viability assay**

The viability of primary neuronal cells was assessed using by MTT assay. Briefly, the primary neuron cells (2×10^4 cells/well) were plated in 96-well culture dishes. After that, 10mL MTT regent were added to each well at 37˚C and incubated for 4 hours. Then, the 100 mL DMSO displace the culture medium to dissolve the formazan crystals. Finally, Absorbance was read by microplate reader (Microplate reader, Bio-Rad, 3550) at 570 nm.

**Electrophysiological patch clamp recording**

Electrophysiological patch clamp recording was using to investigated potential effect of Rb1 on neuronal excitability. Electrophysiological measurements are used to measure the action potential or sodium current of cultured neurons. The electrophysiological patch clamp recording details was performed as previously described[24]. In short, Cell capacitance was measured from a transient current evoked by a -80mV depolarizing step from a holding potential of -70 mV. Then a series of depolarizing current pulses are used and the inherent excitation is checked by constructing an input-output (I-O) function.

**Statistics**

Data were analyzed using the SPSS 19.0 (IBM, Armonk, New York) for Windows covariance software package. Data are presented as mean ± s.e.m. Comparisons between the two groups were performed using the student T test. Statistical differences between groups were assessed using analysis of variance (ANOVA) and Bonferroni postmortem tests. The MWM test was analyzed using bidirectional repeated
measurement (RM) analysis of variance (ANOVA) and Tukey test. Use bidirectional variance analysis to analyze NOR data. The NOR data were analyzed by one-way ANOVA, and then the paired groups (two tails) were tested by student T test. Statistical significance was set as $P<0.05$.

**Results**

**Ginsenoside Rb1 rescues cognitive deficits in APP/PS1 mice**

The cognitive function of APP/PS1 mice was detected the NOR task and MWM test. In the MWM test, there was no statistic difference in escape latency, time spent in target quadrant, the number of target crossings between 2-month-old mice ($p>0.05$) (Fig.1a-e). However, these results showed that the escape latency in 8-month-old APP/PS1 mice was much longer than age-matched WT mice, while treatment with Rb1 was significantly shortened escape latency in APP/PS1 mice ($p<0.05$) (Fig.1 a). The probe tests revealed that 8-month-old APP/PS1 mice spend less time in target zone where hidden platform located during learning period compared with age-matched WT mice, decrease the number of target crossings and percentages of time and distance in the target zone when compared with age-matched WT mice ($p<0.05$) (Fig.1 b-e). Rb1 treatment significantly reduced that difference in APP/PS1 mice ($p<0.05$) (Fig.1 b-e). Besides, we found that the mice in the average swimming speed have not significantly difference in all groups ($p>0.05$) (Fig.1 f).

In NOR task, 2-month-aged mice has not significantly difference in distinguish the familiar and novel objects ($p>0.05$) (Fig.1 g). However, these results showed that 8-month-aged APP/PS1 mice had lower the discrimination index than age-matched WT mice ($p<0.05$) (Fig.1 g). Rb1 treatment significantly reduced the time in distinguish the familiar and novel objects ($p<0.05$) (Fig.1 g).

In a word, these results indicates that Rb1 have an ability to improve the memory decline of APP/PS1 mice.

**Ginsenoside Rb1 may reduce the aberrant hyperexcitability of AD model by regulating the expressions of Nav1.2 and Nav1.6**

As showed in the Fig 3, we investigated the neuronal excitability of APP/PS1 mice and WT mice under different treatments by EEG recording. The EEG recording data showed that it was not visible difference among 2-month-aged mice (Fig. 2). The significantly increased spike-wave discharges (SWDs) and the time in frequency were found in 8-month-aged APP/PS1 mice when compared with the age-matched WT mice, while Rb1 treatment alleviated the abnormal neuronal activity (Fig.2). These finding suggests that Rb1 treatment improve the aberrant neuronal hyperexcitability of APP/PS1 mice.

Followed, to confirm whether Rb1 improves neuronal hyperexcitability by regulating the expression levels of sodium proteins, we assessed the levels of Nav1.2 and Nav1.6, as well as the expression and locations of Nav1.1α. Compared with WT mice, the Nav1.1α level in the cortex or hippocampus of APP/PS1 mice is significantly increase ($p<0.05$) (Fig. 3 a-d), and the level of Nav1.1α in the cortex and the hippocampal
regions extracellularly and intracellularly were also detected (p<0.05) (Fig. 3 a-d). However, Rb1 treatment have not potential to significantly improve the abnormal expression and abnormal position of Nav1.1α in APP/PS1 mice (p>0.05) (Fig. 3 a-d). In additional, we found that the expression of Nav1.2 and Nav1.6 is significantly increase in APP/PS1 mice compared with WT mice, and Rb1 treatment significantly decrease the expression of Nav1.2 and Nav1.6 in in the cortex and hippocampus of APP/PS1 mice (p<0.05) (Fig. 3 a, e-f). In a word, these data support that Rb1 may reduce the aberrant hyperexcitability of APP/PS1 mice, which is related to Nav1.2 and Nav1.6.

**Rb1 attenuated Aβ1-42-induced injury in primary neurons**

As showed in Figure 5, we investigated cell viability of Aβ1-42 (5mM)-treated primary neurons under different experimental conditions. Compared with that of baseline control group, the cell viability was significantly decreased in the cells that were injured by Aβ1-42 at 48h, 72h and 96h (p<0.05) (Fig.4 c-e). There are not significantly different between the blank group and DMSO group (p>0.05) (Fig.4 a-f). These finding implied that neurons showed significant damage after 48 hours of treatment with Aβ1-42. While, we found that the decreased cell viability was restored by Rb1 treatment when compared with DMSO group (p<0.05) (Fig.4 c-d).

**Rb1 reduced the neuronal hyperexcitability and restored sodium current overload induced by Aβ1-42**

Changes in neuronal excitability are known to be key for maintain memory performance in rats [28]. Besides, soluble Aβ1-42 oligomers correlates with neuronal hyperactivity and subsequent affects cognitive deficits and memory dysfunction in AD [2, 29]. As showed in Fig.5, Aβ1-42 induce a significantly increased in the frequency and threshold of neuronal action potentials (AP) (p<0.05) (Fig.5 a-b).

Compared with DMSO group, the frequency, threshold and peak amplitude of the neuronal action potential were not statistical significance in the blank control group (p>0.05) (Fig.6 a-c). Rb1 largely rescued the Aβ1-42-induced increased in the frequency and threshold of neuronal action potentials (p<0.05) (Fig.5 a-b), suggesting that Rb1 have protective role in neuronal hyperexcitability induced by Aβ1-42. However, the peak current density is not significantly different in all groups (p>0.05) (Fig.5 c).

Studies have shown that the increased excitability is partly related to the upregulation of sodium current [30]. Figure 3A showed a typical trace of the total inward current recorded at a negative 60 to positive 60mV voltage step. Not surprisingly, Aβ1-42, which play major neurotoxicity roles in AD, gave rise in a largely decreased in sodium current density curves and peak current density of neurons (p<0.05) (Fig.5 d-e). Importantly, Rb1 reversed the decrease induced by Aβ1-42 (p<0.05) (Fig.5 d-e).

These results indicates that Rb1 have protective role in neuronal hyperexcitability and sodium current overload induced by Aβ1-42.

**The neuroprotective effect of Rb1 on Aβ1-42 may be related to the regulation of Nav1.2 and Nav1.6**
To determine whether the protection of Rb1 on Aβ1-42-induced neuronal hyperexcitability and sodium current overload was due to the sodium channel proteins, we detected the level of Nav1.2 and Nav1.6, as well as the expression and location of Nav1.1α.

These data showed that Aβ1-42 the expression of Nav1.2, Nav1.6, total Nav1.1α and intracellular Nav1.1α significantly increased after Aβ1-42 treatment when compared to the baseline control group (p<0.05) (Fig.6 a-b, d-f), while the levels of extracellular Nav1.1α was significantly reduced (p<0.05) (Fig.6 c). Besides, the levels of Nav proteins were not statistical significance when compared the blank group and the DMSO group (p>0.05) (Fig.6 a-f). After Rb1 treatment, the increased expressions of Nav1.2 and Nav1.6 induced by Aβ1-42 were significantly reduced (p<0.05) (DMSO group vs. Rb1 group) (Fig.6 a, e-f). However, there were no statistical significance in the expressions and location of Nav1.1α between the DMSO and Rb1 group (p>0.05) (Fig.6 a-d). Our finding demonstrates that the Rb1-induced protective roles in neuronal hyperexcitability might have relational with the regulation in Nav1.2 and Nav1.6.

**Discussion**

Our knowledge of the effects underlying the protection of ginsenoside Rb1 in AD remains limited despite the evidence pointing to a positive effect of PNS in AD [31]. We investigated that the neuronal excitability restoring attributes of ginsenoside Rb1 on the APP/PS1 mice and AD model *in vitro*. Our study clearly showed that Rb1 significantly improved cognitive and memory impairment of APP/PS1 mice and partially reversed the abnormal hyper-excitability of APP/PS1 mice. Moreover, the expressions of Nav1.2 and Nav1.6 in APP/PS1 mice after treated with ginsenoside Rb1 were significantly decreased as compared with APP/PS1 mice. Furthermore, we observed that Rb1 had a neuroprotective effect on Aβ1-42-injured neurotoxicity as displayed by the alleviation of the decreased cell viability and excitability of primary neurons. The mechanism of its neuroprotective effects may be involved in regulating the voltage-gated sodium current and neuronal excitability.

The administration of Aβ1-42 in primary neurons was verified as AD model *in vitro*, which was developed to AD research. Increasing evidences showed that Aβ1-42 peptides plays important role in the pathogenesis of AD [32], which were confirmed to induce neuronal dysfunction [33]. Moreover, high level of Aβ is associated with aberrant excitatory network activity, thus resulting in cognitive decline [34]. Further, it is believed that neurons are the primary source of Aβ1-42[34]. In our study, we noticed that increased Aβ1-42 treatment changed the expression of sodium channel proteins significantly, such as Nav1.1α, Nav1.2 and Nav1.6. In fact, Aβ peptide can induce the depolarization of skeletal muscle plasma membranes and therefore cause the motor dysfunction by increasing Na+ influx [35]. In addition, previous study found that GABAergic inhibitory transmission played roles in Aβ1-42-induced hyper-excitability [36]. Consistent with previous studies, our study showed that the cell viability of neuron after treated with Aβ1-42 was significantly decreased and the neuronal excitability appeared to partially restore [37, 38]. Furthermore, the expression of sodium channel proteins was significantly different after treatment with Aβ1-42. However, the current study showed that Rb1 significantly decreased the Aβ1-42-induced neuron injury and abnormal neuron hyper-excitability.
AD, a form of epilepsy, is characterized by increased neuro-excitability [39]. Studies have shown that abnormal nerve excitability and pathological hyperexcitability are related to cognitive deficits [40]. In this study, the effects of Rb1 on cognitive and memory is estimated by MWM and NOR test. We found that treatment with ginsenoside Rb1 improved the cognitive and memory function of APP/PS1 mice. Moreover, EEG tests indicated that the aberrant neuronal excitability was reversed by ginsenoside Rb1. In an attempt to investigate mechanisms underlying changes in excitability, we analyzed the neuronal sodium voltage-gated channels. Nav1.2 and Nav1.6, play roles in control the generation and propagation of action potentials in neurons, are widespread in the hippocampus and cortex [41-44]. Previous research indicated that PNS displayed protective effects against cognitive impairment of senescence-accelerated mice prone 8 (SAMP8) through mechanisms possibly including reduction of sodium channel-voltage-gated-beta 2 (SCN2B) expressions[23]. Consistent with the results, we proposed that ginsenoside Rb1 might be involved in regulating Nav proteins and redressing the aberrant neuronal hyperexcitability induced by Aβ1-42. In this study, the expressions of Nav1.2 and Nav1.6 significantly reversed by Rb1 treatment in the cortex and hippocampus of APP/PS1 mice. In summary, our work revealed a novel mechanism involved in modulation of Rb1, as proved by decreased the expression of Nav1.2 and Nav1.6, thus decreased the neuronal hyperexcitability of APP/PS1 mice.

**Conclusions**

In conclusion, ginsenoside Rb1 have neuroprotective effect and rescues learning and memory deficits in APP/PS1 mice by regulating the excitability, which may is associating with the expression of Nav1.1 and Nav1.6. Meanwhile, our work showed that ginsenoside Rb1 play a key role in against Aβ1-42-induced neurotoxicity.

**Declarations**

**Ethics approval**

All the experiments and animal care complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23; revised 1996). This study was performed in accordance with the Care and Use Guidelines of Experimental Animals established by the Research Ethics Committee of Kunming University of China (permit no. kmu-eac-2018021; Kunming, China). Isoflurane was used for inducing anesthesia for the surgical procedures. The purchase, storage and use of the isoflurane were approved by Kunming Medical University (license no. kmykdx-D-A00272; Kunming, China).

**Consent for publication**

Written informed consent for publication was obtained from all participants.

**Availability of data and material**
The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a possible conflict of interest.

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Authors' contributions:

Shan Li, Yan-Bin XiYang and Min-Nan Lu: Conceptualization, Methodology, Formal analysis, Writing - Review & Editing, Supervision. Qian Wang, Xiao-Min Zhang, Hao-Ran Zhao and Yun Yuan: Investigation, Writing – Original Draft, Project Administration. Wan-Hai Ding, Wen-Wei Gao, Xu-Yang Wang and Shi-Wen Chen, Xiao-Han Dong, Guo-Ji Yan: Investigation, Data Curation.

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