Panax Notoginseng Saponins attenuates sevoflurane-induced nerve cell injury by modulating AKT signaling pathway

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Abstract. General anesthesia in patients with or at risk for neuronal injury remains challenging due to the neurotoxic effects of volatile anesthetics. One inhalation anesthetic, sevoflurane, induces neuronal damage, including neuroapoptosis, and learning and memory impairment. Panax Notoginseng Saponins (PNS) is the active ingredient of Sanqui and has been reported to exert neuroprotective effects. In the current study, the protective effect of PNS on sevoflurane-induced nerve cell injury was explored. Cell proliferation was significantly reduced in a dose-dependent manner following stimulation with sevoflurane. Furthermore, cell apoptosis and the protein expression of caspase-3, caspase-9 and Bax were significantly increased, while the expression of Bcl-2 was decreased in the sevoflurane group compared with normal control. Furthermore, the protein level of Bace-1, APP and Aβ were elevated in the sevoflurane group compared with the control group. By contrast, PNS treatment significantly reduced the neurotoxicity induced by sevoflurane. Additionally, sevoflurane reduced activation of the AKT signaling pathway, which was activated by PNS treatment. In conclusion, the results suggested that PNS attenuates sevoflurane-induced neurotoxicity through by stimulating cell proliferation and inhibiting cell apoptosis. These effects were mediated, at least in part, by activating the AKT signaling pathway.

Introduction

An estimated 200 million patients undergo anesthesia and surgery worldwide each year (1). Volatile anesthetics, such as isoflurane, desflurane and sevoflurane, are usually used in general anesthesia. The differences in the chemical structures of volatile anesthetics result in diverse physicochemical properties leading to different biological effects, with particularly different effects on neuronal cells (2). Depending on the experimental conditions used, sevoflurane can be neuroprotective in anesthesia (3). However, in certain conditions, particularly in animal models with neonatal sevoflurane exposure, sevoflurane can exert relevant neurotoxicity effects (4). Additionally, increasing studies have demonstrated that exposure to individual anesthetic drugs, including volatile anesthetics, triggers significant damage in the developing brain (5). Furthermore, sevoflurane has been reported to induce cell damage in various neuronal and non-neuronal cells and tissues (6). Thus, volatile anesthetic, such as sevoflurane, can be a risk for cell injury, particularly for neuronal injury during anesthesia. Therefore, understanding the pathological mechanisms of the neurotoxic effects of sevoflurane is of great importance for developing effective methods of anesthesia.

Traditional Chinese medicine has been developed in China over 5,000 years, providing health care services to Chinese people and worldwide. An *in vitro* study demonstrated that traditional Chinese medicine may be effective for the treatment and prevention of central nervous system diseases (7). Thus, the effect of traditional Chinese medicine on nerve cell protection has become a major topic of research in the Chinese neuroscience community, and an important part of medical research worldwide.

Panax Notoginseng Saponins (PNS) is the active ingredient of the Chinese herb Sanqui, which is predominantly cultivated in the Yunnan and Guangxi provinces of China (8). The medicinal properties of the Panax Notoginseng root include relieving swelling, promoting blood clotting and alleviating pain (9). It has been reported that PNS has a number of biological activities including immunomodulatory effects, antioxidation and anticancer properties (10). Additionally, it has been demonstrated that PNS has neuroprotective effects following stroke through reducing the apoptosis of nerve cells and neurotoxicity (11). Another study suggested that PNS promoted angiogenesis and the synthesis and release of neurotrophic factors (12). However, the effect of PNS on anesthesia-induced neurotoxicity effects remains to be elucidated.

In the present study, nerve cells were separated from the hippocampus of day 16 embryonic mice and used to investigate

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Abbreviations: PNS, Panax Notoginseng Saponins; APP, amyloid precursor protein; Aβ, β-amyloid peptide

the influence of PNS on sevoflurane-induced nerve cell injury. By culturing the neuronal cells in a sevoflurane environment using an anesthesia machine, it was determined that sevoflurane significantly induced neurotoxicity by decreasing cell growth and increasing apoptosis. Additionally, PNS treatment inhibited the neurotoxic effect of sevoflurane. Furthermore, PNS attenuated sevoflurane-induced neurotoxicity through the phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) pathway. Understanding the mechanism of sevoflurane-induced neurotoxicity is essential for providing novel insights into the action of volatile anesthetics and developing neuroprotective strategy for anesthesia-induced neuronal injury.

Materials and methods

Animals. This study was approved by the ethical committee of the Experimental Animal Center of Harbin Medical University (Harbin, China). All experimental animals were purchased from the Experimental Animal Center of Harbin Medical University. All experiments with animals were performed according to the guidelines of the University Ethics Committee of Harbin Medical University. Neuronal cells were harvested from embryonic day 16 mice by caesarean section from pregnant BALB/c mice and derived from the hippocampus. The harvested cells were first plated on 24 or 96-well plates pre-coated with poly-L-lysine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and cultured at 37°C with 5% CO_2. The cells were cultured in neurobasal medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with B27 (X1) and glutamine (25 mM; Sigma-Aldrich; Merck KGaA). After 7 days, the neuronal cells were ready to use for the following experiments.

Treatment of sevoflurane and PNS. The neuronal cells were first divided into four groups for sevoflurane (Sigma-Aldrich; Merck KGaA) treatment: Normal group, cells cultured in 95% O_2 and 5% CO_2; and sevoflurane groups, cell cultured in 95% O_2 and 5% CO_2 with 1, 2 or 3% sevoflurane. All gases were delivered into cells using an anesthesia machine in a sealed plastic box. For the PNS (Sigma-Aldrich; Merck KGaA) experiments, the cell were divided into five groups: Normal group, normal neuronal cells; 3% sevoflurane group, cells treated with 95% O_2 and 5% CO_2 with 3% sevoflurane for 6 h; PNS groups, cells cultured in 50, 100 or 200 μmol/l PNS for 6 h and then with 3% sevoflurane for 6 h.

MTT assay. The cell growth and viability were assessed by an MTT assay. Following the addition of growth medium containing 10% 5 mg/ml MTT (Sigma-Aldrich; Merck KGaA), the cells were seeded in a 96-well plate and cultured at 37°C overnight in 5% CO_2. Then, the formazan crystals were dissolved with dimethylsulfoxide. Optical density was determined using a microculture plate reader (BD Biosciences, Franklin Lakes, NJ, USA) at 490 nm.

Cells apoptosis analysis. Cell apoptosis was detected using flow cytometric analysis using an Annexin V-FITC/propidium iodide kit (Sigma-Aldrich; Merck KGaA). Briefly, cells were trypsinized and washed with phosphate buffered saline. After

centrifugation at 300 x g for 10 min at room temperature, the cell were resuspended in 500 μl of binding buffer, cells were incubating with 5 μl Annexin V-fluorescein isothiocyanate and 5 μl propidium iodide (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Ultimately, all specimens were analyzed on a FACScan flow cytometer with CellQuest Pro software 5.1 (BD Biosciences).

Western blot analysis. The protein expression level was assessed by western blot. Total protein from neuronal cells was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Nantong, China) and quantified using a bicinchoninic acid assay. Proteins (200 μg) were separated by 10 or 15% SDS-PAGE and transferred to nitrocellulose membranes. Following blocking in a 5% skimmed milk solution for 30 min at room temperature, the target proteins were incubated overnight at 4°C with anti-capase-3 (1:500; ab13847), anti-capase-9 (1:2,000; ab202068), anti-B cell lympoma-2 (Bcl-2; 1:10,000; ab59348), anti-Bcl-2 associated X protein (Bax; 1:1,000; ab32503), anti-β-secretase (Bace-1; 1:1,000; ab183612), anti-phospho-AKT (1:500; ab38449), anti-AKT (1:500; ab8805) or anti-β-actin (1:1,000; ab8827) rabbit anti-mouse antibodies (all from Abcam, Cambridge, UK). Membranes were subsequently incubated with a goat anti-rabbit secondary antibody (1:20,000; ab7090; Abcam, Cambridge, UK) for 1 h at room temperature. The band density of each gene was normalized to the corresponding density of β-actin and visualized using the enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Little Chalfont, UK). Band densities were quantified using Odyssey Image Analysis software version 4.0 (LI-COR Biosciences, Lincoln, NE, USA).

ELISA. The content of amyloid precursor protein (APP; E2035m; Beijing Huaxia Tech, Beijing, China) and β-amyloid peptide (Aβ; KMB3441; Invitrogen; Thermo Fisher Scientific, Inc.) concentrations were measured using corresponding quantification ELISA kits according to the manufacturer's instructions. Optical density values were read at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell transfection. AKT siRNA was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences were as follows: AKT siRNA sense, GCCAGUACCUCAUGGAUU ATT and antisense, UAAUCCAUGAGGUACUGGCTT; siRNA control sense, GGACTATCATATGCTTACCGAA and antisense, CAGGAAACAGCTATGACG. Additionally, neuronal cells were divided into four groups. Control group, normal neuronal cells; 3% sevoflurane group, cells treated with 95% O_2 and 5% CO_2 with 3% sevoflurane; 3% sevoflurane + PNS group, cells treated with 200 μmol/l PNS and then 3% sevoflurane; 3% sevoflurane + PNS + siAKT group, cells were transfected with AKT siRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h and then treated as the PNS group.

Statistical analysis. All results were presented as the mean ± standard deviation from a minimum of three replicates. Differences between groups was evaluated using SPSS version 15.0 statistical software (SPSS, Inc., Chicago. IL, USA) with
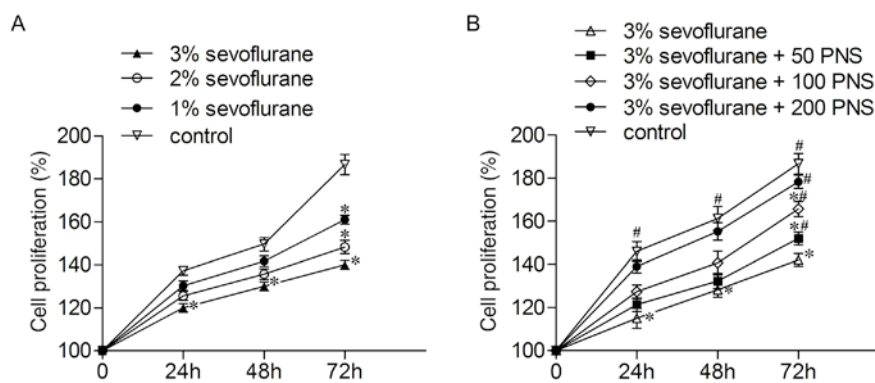
Figure 1. PNS elevates cell proliferation in sevoflurane-stimulated nerve cells. (A) Nerve cells were divided into four groups. Control group, normal cells; sevoflurane groups, cells cultured in 95% O_2 and 5% CO_2 with 1, 2 or 3% sevoflurane. The cell proliferation rates were measured by MTT assay. *P<0.05 vs control group. (B) Nerve cells were divided into five groups. Control group, normal cells; 3% sevoflurane group, cells cultured in 95% O_2, 5% CO_2 and 3% sevoflurane; 3% sevoflurane + PNS groups, cells treated with 50, 100 or 200 μmol/l PNS for 6 h and then with 3% sevoflurane. The cell proliferation was detected by MTT assay. *P<0.05 vs control group, #P<0.05 vs. 3% sevoflurane group. Data are presented as the mean ± standard deviation. PNS, Panax Notoginseng Saponins.
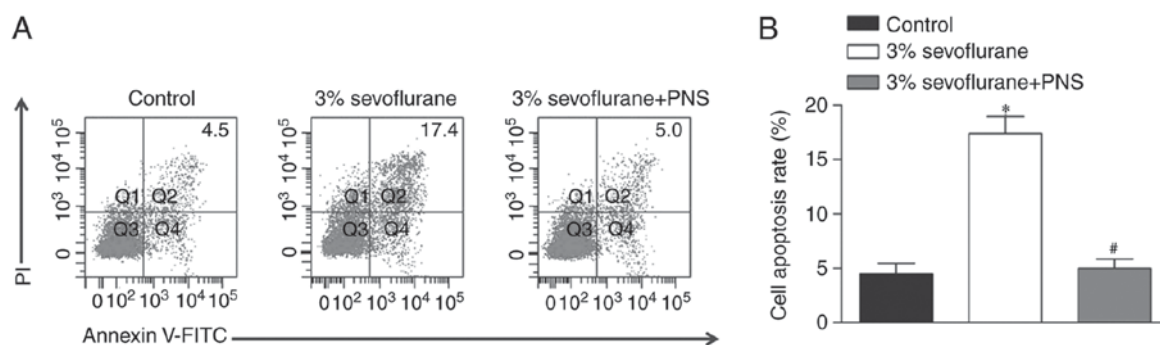

Figure 2. PNS protects nerve cells against sevoflurane-induced apoptosis. The nerve cells were divided into three groups. Control group, normal cell; 3% sevoflurane group, cells delivered with 3% sevoflurane; 3% sevoflurane + PNS group, cells treated with 200 μmol/l PNS for 6 h and then with 3% sevoflurane for 6 h. (A) Cell apoptosis was measured by flow cytometry assay. (B) Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. 3% sevoflurane group. PNS, Panax Notoginseng Saponins; PI, propidium iodide; FITC, fluorescein isothiocyanate.

one-way analysis followed by a Bonferroni post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

PNS elevates cell proliferation in sevoflurane-stimulated nerve cells. To explore the effect of sevoflurane on nerve cell proliferation, the cells were treated with 1, 2 and 3% sevoflurane. As presented in Fig. 1A, the proliferation rates of the nerve cells were inhibited by sevoflurane in a concentration-dependent manner. Given the effectiveness of the sevoflurane at these concentrations (1, 2 and 3%), 3% sevoflurane was used to perform the subsequent experiments. The nerve cells were cultured in the presence of increasing concentrations of PNS (50, 100 and 200 μM) then treated with 3% sevoflurane, and this led to an increase in cell proliferation in a concentration-dependent manner (Fig. 1B); thus PNS at 200 μM was used in the subsequent experiments. These results demonstrated that PNS alleviates reduced cell proliferation induced by sevoflurane treatment in nerve cells.

PNS protects nerve cell against sevoflurane-induced apoptosis. To investigate the effects of PNS on sevoflurane-stimulated nerve cells, the cell apoptosis of nerve cells was examined using flow cytometric analysis. Following treatment with 3%

sevoflurane, the nerve cells were stimulated with 200 μM PNS. The results illustrated that the treatment with sevoflurane induced an increase in apoptosis rate, whereas PNS inhibited the apoptosis rate in nerve cells compared with those treated with sevoflurane only (Fig. 2).

PNS regulates the expression of cell apoptosis-associated proteins in sevoflurane-stimulated nerve cells. To further validate the regulatory effect of PNS on sevoflurane-induced nerve cell apoptosis, the expression level of caspase-3, caspase-9, Bax and Bcl-2 were determined using western blot (Fig. 3). The results demonstrated that the expression of caspase-3, caspase-9 and Bax were significantly increased in the sevoflurane group as compared with control group, and decreased in sevoflurane + PNS group compared with the sevoflurane group. Furthermore, the expression level of Bcl-2 was decreased in the sevoflurane group compared with control group, and elevated by PNS treatment. These results further confirmed PNS inhibited cell apoptosis in sevoflurane-stimulated nerve cells.

PNS suppresses the expression bace-1, APP and Aβ in sevoflurane-stimulated nerve cells. To evaluate the effect of PNS on neurotoxicity, the expression level of Bace-1 was detected using western blot in sevoflurane-stimulated nerve cells. As
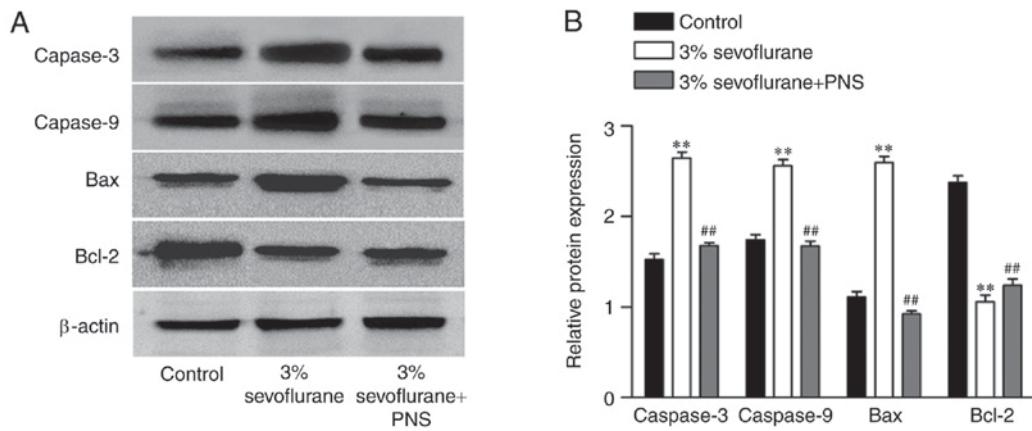
Figure 3. PNS regulates cell apoptosis-associated proteins in sevoflurane-stimulated nerve cells. (A) Protein expression levels of caspase-3, caspase-9, Bax and Bcl-2 were measured by western blot. (B) Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. Data are presented as the mean ± standard deviation. $^{**}P<0.01$ vs. control group; $^{\#\#}P<0.01$ vs. 3% sevoflurane group. PNS, Panax Notoginseng Saponins; Bax, Bcl-2 associated X apoptosis regulator; Bcl-2, Bcl-2 apoptosis regulator.
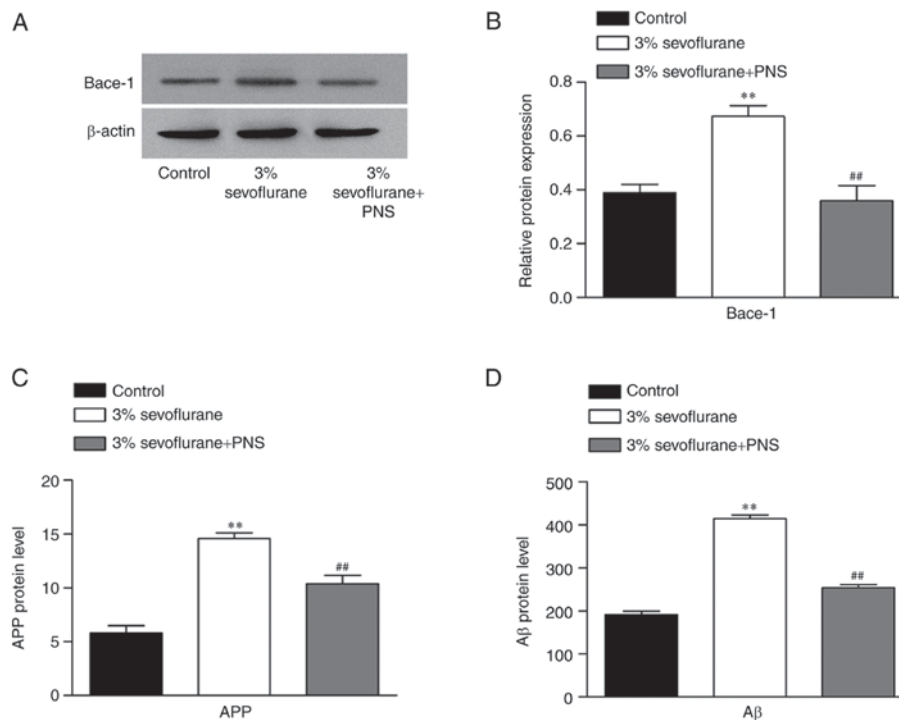

Figure 4. PNS suppresses the expression Bace-1, APP and Aβ in sevoflurane-stimulated nerve cells. (A) Protein expression levels of Bace-1 was measured by western blot. (B) Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. The protein level of (C) APP and (D) Aβ were detected by ELISA method. Data are presented as the mean ± standard deviation. $^{**}P<0.01$ vs. control group; $^{\#\#}P<0.01$ vs. 3% sevoflurane group. PNS, Panax Notoginseng Saponins; Bace-1, β-secretase 1; APP, amyloid precursor protein; Aβ, β-amyloid peptide.

presented in Fig. 4A and B, the expression of Bace-1 was significantly increased in the sevoflurane group as compared with control group, and inhibited by PNS treatment compared with the sevoflurane group. Additionally, the protein level of APP and Aβ were measured by ELISA (Fig. 4C and D). The results demonstrated that the protein levels of APP and Aβ were significantly increased in the sevoflurane group, and decreased by PNS treatment, which was consistent with the changes in the protein expression of Bace-1.

PNS elevates sevoflurane-inhibited AKT signaling in nerve cells. In the present study, the role of AKT signaling in

sevoflurane-induced nerve cell injury and the protective effects of PNS were examined using western blot. The results demonstrated that the phosphorylation of AKT was significantly decreased by sevoflurane, and the levels of total AKT showed no changes. Additionally, the phosphorylation of AKT was restored following the treatment with PNS. To further confirm the role of AKT, the nerve cells were transfected with a specific siRNA targeting AKT and the control cells were transfected with siRNA control sequences. Moreover, the results suggested that the protective effects of PNS were markedly diminished by AKT siRNA transfection. These results suggested that PNS protected against
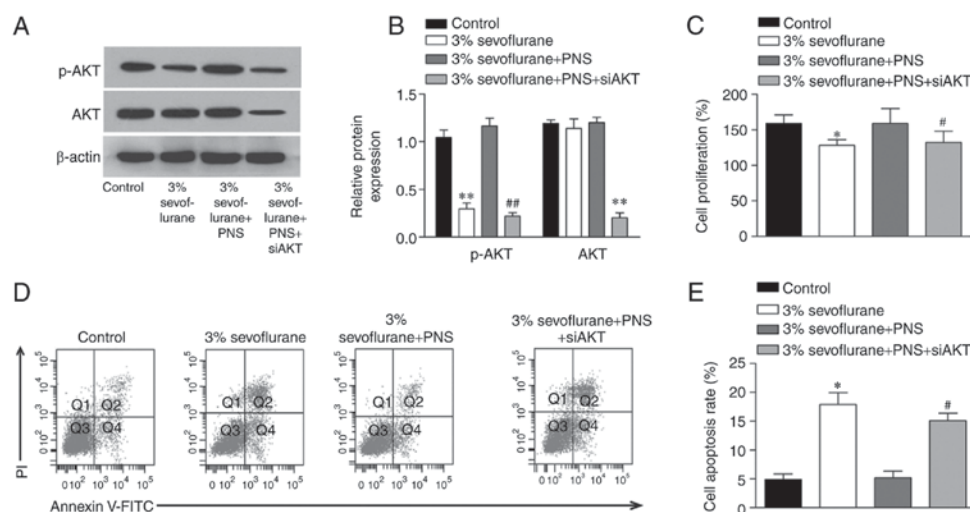
Figure 5. PNS elevates sevoflurane-inhibited AKT pathway in nerve cells. The nerve cells were divided into four groups. Control group, normal cell; 3% sevoflurane group, cells delivered with 3% sevoflurane; 3% sevoflurane + PNS group, cells treated with 200 μmol/l PNS for 6 h and then delivered with 3% sevoflurane; 3% sevoflurane + PNS + siAKT group, cells were transfected with siAKT for 48 h then treated as PNS group. (A) Protein expression levels of p-AKT and AKT were measured by western blot. (B) Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. (C) Cell proliferation was detected at 48 h after treatment using MTT method. (D) Cell apoptosis was measured by flow cytometry assay. (E) Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. 3% sevoflurane + PNS group. p-, phosphorylated; AKT, AKT serine/threonine kinase; PNS, Panax Notoginseng Saponins; siAKT, AKT-targeting small interfering RNA.

sevoflurane-induced nerve cell injury by promoting the AKT signaling pathway (Fig. 5).

Discussion

Anesthesia neurotoxicity in the developing brain has become a major health issue of interest to the medical community and the public (13). Sevoflurane is a commonly used inhalation anesthetic. Previous studies have reported that anesthesia with sevoflurane can induce neurotoxicity in the brain tissues in adult mice, and in fetal and offspring mice (14). PNS, extracted from *Panax notoginseng*, a perennial herb of a perennial herb of the *Acanthopanax gracilistylus* family, inhibits neuronal apoptosis, inflammation and focal ischemia, therefore it may be beneficial in the treatment of nerve injury (12). However, to the best of our knowledge, no reports to date have investigated the effect of PNS on sevoflurane-induced neurotoxicity. In the current study, the neuroprotective effects of PNS against sevoflurane-induced neurotoxicity were investigated *in vitro* systems. The results demonstrated that administration of PNS protected nerve cells against sevoflurane-induced neurotoxicity, increasing cell proliferation and inhibiting apoptosis. Further investigating demonstrated that PNS promoted AKT signaling in the sevoflurane-stimulated nerve cells.

Although the underlying molecular mechanisms of neurotoxicity are not yet fully understood, altered cell proliferation and apoptosis have been implicated. A previous study have demonstrated that inhalation anesthetic induces widespread cerebral neuroapoptosis in neonatal rat pups with subsequent long-term neurocognitive impairment of the animals (15). Another finding suggested that the sevoflurane may induce neurotoxicity *in vitro* (16). A previous study reported that anesthesia with 2.5% sevoflurane for 2 h can induce neurotoxicity in the brain tissues of adult mice (17). In the current study, nerve cells were treated with sevoflurane *in vitro* at the concentrations of 1, 2 and 3%. The results demonstrated

that the cell proliferation was decreased by sevoflurane stimulate. Furthermore, the neurotoxic effect was stronger at higher concentrations of sevoflurane, and the cell growth as significantly reduced by stimulation with 3% sevoflurane. These results were consistent with the previous study by Satomoto *et al* (18). Thus, 3% sevoflurane was used to perform subsequent experiments, and the results demonstrated that sevoflurane significantly elevated cell apoptosis. These results suggested that sevoflurane has a neurotoxic effect on nerve cells *in vitro* by inhibiting cell proliferation and promoting cell apoptosis.

PNS has become one of China's fastest-growing drugs used in hospitals. Injectable preparations of Radix notoginseng, including freeze-dried Xueshuantong and Xuesetong powders, have been used in the clinic for maintenance treatment of acute cerebral infarction and its complications (19). A previous study have demonstrated the beneficial effects of PNS on central nervous system disorders and neurodegenerative diseases, thus suggesting a neuroprotective role of PNS (20). In the current study, the effect of PNS on sevoflurane-stimulated nerve cells was investigated. The results indicated that administration of PNS to sevoflurane-induced nerve cells significantly elevated cell proliferation. Additionally, the effect was most obvious in the 200 μM PNS group. Antioxidant, anti-inflammatory and anti-apoptotic activities have previously been suggested as mechanisms underlying the therapeutic effects of PNS (21). The present study demonstrated that PNS restrained sevoflurane-induced nerve cell apoptosis, which was consistent with previous research (21). These results suggested that PNS treatment inhibited the neurotoxic effect of sevoflurane in nerve cells.

It has been reported that excessive Aβ accumulation is a major pathological hallmark of neurological disorders (14). Aβ is produced via serial proteolysis of the APP protein by Bace-1 enzyme. Increasing evidence suggested that the caspase activation and apoptosis in nerves may enhance the Bace-1 level

and then facilitate APP processing, leading to increases in Aβ levels (22). In the present study, the results illustrated that sevoflurane increased the expression levels of Aβ, Bace-1 and APP, while PNS treatment decreased the protein expression levels. These results suggested that PNS treatment inhibited sevoflurane-induced Aβ accumulation and attenuated the progression of neurological dysfunctions.

An earlier study reported that PNS alters the activity of PI3K/AKT signaling pathway molecules, and then regulated the cell proliferation and apoptosis (23). In the current study, the results indicated that nerve cells stimulated with sevoflurane had significantly decreased phosphorylation of AKT. In addition, PNS increased cell proliferation and inhibited cell apoptosis. However, the protective effects of PNS were markedly diminished by a specific siRNA targeting AKT. These results suggested that PNS effectively protects nerve cells from sevoflurane-induced cytotoxicity by activating the AKT signaling pathway.

In conclusion, the present study provides strong evidence that PNS regulates neurotoxicity triggered by sevoflurane *in vitro*. Experiments using cell culture revealed that PNS acted, at least in part, by activating the AKT signaling pathway. These findings provide a novel theory supporting current clinical experiments aimed at assessing the beneficial effects of PNS administration against sevoflurane-induced cytotoxicity.

Acknowledgements

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