Vitexin improves neuron apoptosis and memory impairment induced by isoflurane via regulation of miR-409 expression

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Abstract

Background. Anesthetics, such as isoflurane, sevoflurane, ketamine, and desflurane, are commonly used in clinics. Specifically, isoflurane is one of the most commonly used inhalational anesthetics, which can be used in surgery patients of all ages, including children.

Objectives. The aim of the study was to investigate the mechanisms of vitexin against isoflurane–induced neurotoxicity.

Material and methods. Reference memory testing was performed for 5 days (4 trials, 2 per day) before anesthesia. Reversal testing was performed on the 3rd day after anesthesia. The cell viability and apoptosis of PC-12 cells were detected using MTT and TUNEL assays, respectively. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), glutathione (GSH), and superoxide dismutase (SOD) concentrations. The concentration of reactive oxygen species (ROS) was detected using ROS measurement. Expression of miR-409 was determined using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Protein expression levels were detected using western blotting.

Results. Rats treated with isoflurane showed significant increases in the escape latency periods (ELP) and the apoptosis of hippocampus neuron cells; this effect was reversed by 3 mg/kg or 10 mg/kg of vitexin (p < 0.05). Further testing showed that isoflurane could significantly decrease the cell viability and increase the apoptosis of PC-12, the expression of inflammatory cytokines (TNF-α and IL-6) and ROS (p < 0.05). However, these results were reversed by 10/100 μM of vitexin. In addition, vitexin could significantly increase the expression of miR-409 (p < 0.05). Further studies showed that overexpression of miR-409 could significantly promote the effect of vitexin on isoflurane–induced neurotoxicity (p < 0.05). Finally, overexpression of miR-409 could significantly increase the expression of p-AMPK/t-AMPK and p-GSK3β/t-GSK3β.

Conclusions. Vitexin has protective effects against isoflurane–induced neurotoxicity by targeting miR-409 and the AMPK/GSK3β pathway.

Key words: neurotoxicity, isoflurane, vitexin, miR-409, AMPK/GSK3β signaling pathway
Each year, more than 2 billion people around the world need surgical treatment. Anesthetics such as isoflurane, sevoflurane, ketamine, and desflurane are commonly used in clinics. Specifically, isoflurane is one of the most commonly used inhalational anesthetics, which can be used in surgery patients of all ages, including children. However, recent studies have shown that isoflurane has a correlation with postoperative cognitive dysfunction. Further experimental results demonstrate that isoflurane is neurotoxic, with effects like increasing apoptotic neurons, reducing neurogenesis and changing the ultrastructure of synapses in the central nervous system, including the hippocampus, thalamus, cortex, and spinal cord of rats in the developmental period. Aged neurons are particularly vulnerable to isoflurane. It has also been confirmed that isoflurane can cause cognitive dysfunction persisting for several weeks after treatment in adult and aged rats. Researchers have demonstrated that general anesthesia might contribute to cognitive deficits after surgery, especially in elderly patients. It has also been found that isoflurane anesthesia can cause long-term behavioral changes in the developmental period. These studies indicate that the neurotoxic effects of isoflurane anesthesia might be a risk factor increasing the likelihood that children will develop learning disabilities or deviant behavior in later life. For all these reasons, it is particularly important to seek drugs that can effectively prevent or eliminate these neurotoxicity effects.

It has been reported that flavonoids extracted from hawthorn can lower blood pressure and lipid peroxidation, and increase coronary flow, resulting in protective effects on myocardial ischemic injury. In addition, it has also been revealed that flavonoids have beneficial effects on memory and learning. In 2000, Commenges et al. showed that flavonoids play an important role in preventing human dementia. It has also been reported that flavonoids inhibit acetylcholinesterase activity, which can promote neuron development and nerve regeneration. It has been shown that flavonoids perform a regulative role through selective actions at different signaling cascades, such as PI3 kinase (PI3K)/Akt, protein kinase C (PKC), tyrosine kinase and mitogen-activated protein kinase (MAP kinase) signaling pathways. There is also some evidence that flavonoids interact with the genes involved in mitogen-activated protein kinase (MAPK) signaling pathways, which have also been confirmed to be involved in mediating neuronal survival, regeneration and apoptosis.

Vitexin, a flavone glycoside isolated from hawthorn leaves, is attributed with various medicinal properties, such as lowering blood pressure, reducing inflammation and inhibiting tumors. It has been reported that hawthorn leaves may possess cardiotonic, antiarrhythmic, antiangiinal, and antioxidative functions, and that it may reduce the symptoms of acute myocardial ischemia. In the present study, the apoptosis of hippocampus neuron and memory deficits in rats were investigated. Meanwhile, in vitro explorations were also conducted to reveal the role of miR-409 in neuronal apoptosis. PC-12 cells are a clonal cell line derived from rat pheochromocytoma. They have been widely used as models of both adrenal chromaffin cells and sympathetic neurons. These cells are also critical to in vitro explorations of neuronal cell behavior due to their reversible adoption of several neuronal characteristics upon exposure to nerve growth factor (NGF). In addition, these cells are vulnerable to anesthetic-induced neurotoxicity. Therefore, we chose PC-12 cells as the experimental model. Our study might provide new insights into therapeutic targets for the treatment of isoflurane-induced neurotoxicity.

Material and methods

Animals

A total of 50 male Sprague Dawley rats (200–250 g) were obtained from the Experimental Animal Center of the Central Hospital of Cangzhou, China, and were housed in groups of 4 per cage under standard laboratory conditions. They were maintained at constant room temperature (21 ±2°C) under a normal 12 h light/12 h dark (12L:12D) regime with free access to food and water. All the animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) to minimize the number of animals and their suffering.

Experimental procedure

The rats were randomly divided into 5 groups of 10 animals each: group 1 – the control group; group 2 – an isoflurane-treated group; group 3 – a 1.5 mg/kg vitexin-treated group; group 4 – a 3 mg/kg vitexin-treated group; and group 5 – a 100 mg/kg vitexin-treated group. The isoflurane- and vitexin-treated groups were exposed to 1.4% isoflurane (Sigma-Aldrich/Merck Millipore, Darmstadt, Germany) in a 100% oxygen environment for 2 h. Following isoflurane treatment, the vitexin-treated groups additionally received 1 mg/kg, 3 mg/kg and 10 mg/kg vitexin (Sigma-Aldrich/Merck Millipore) for 30 min.

Learning and memory testing

Before and after exposure to the anesthetics, learning and memory tests were performed on the rats in a Morris water maze (MWM). The MWM was 150 cm in diameter and was filled with opacified water (22 ±1°C) to the height of 1.5 cm above the top of a movable 15 cm diameter platform. The rats were tracked with a video camera mounted above the pool connected to a computer running IMAQ PCI-1407 software. The time between trials was at least 60 min. Reference memory tests were performed for 5 days (4 trials, 2 per day) before anesthesia. In accordance with an earlier study, reversal tests were performed on the 3rd day after anesthesia.
Reference memory test

For all trials, the platform was placed in the target quadrant and the rats started in a random quadrant. The maximum swimming time was 120 s; if rats failed to find the hidden platform in 120 s, they were gently guided to the platform and remained on the platform for 10–15 s. The time to reach the platform (escape latency period (ELP)), and the swimming speed were recorded for each trial.

Reversal testing

The platform was moved to the opposite quadrant of the pool but all distal visual cues remained consistent. Preceding the start of the test, the animals were placed on the platform for 30 s, then removed from the pool. For the test they were placed in the pool and swam to locate the platform in the new target quadrant. The maximum swimming time was set at 120 s for each of 3 trials. Escape latency period and the swimming speed were recorded for each trial. Reversal learning measured how quickly an animal is able to erase their initial learning of the platform’s position and acquire a direct path to the new location.

Following these tests, the rats were euthanized by decapitation under anesthesia.

Cell culture

Human PC-12 pheochromocytoma neurosecretory cells were induced to differentiate by NGF and cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone; GE Healthcare Life Sciences, Logan, USA) containing 9% heat-inactivated fetal calf serum (Invitrogen/Thermo Fisher Scientific Inc., Carlsbad, USA), 100 µg/mL streptomycin, 100 U/mL penicillin and 2 mM L-glutamine (Thermo Fisher Scientific Inc.), and were maintained at 37°C in 5% CO₂ with 95% humidity.

Cell treatment and viability analysis

Cells were seeded at a density of 1 × 10⁴ cells per well in 96-well plates before being exposed to 2% isoflurane for 12 h, and then cultured with 1 µM, 10 µM and 100 µM vitexin for 24 h. The PC-12 cells were treated with 2',7'-dichlorofluorescein diacetate for 6 h, then incubated with cell lysis buffer (OxiSelect ROS assay kit; Cell Bioslabs, Inc., San Diego, USA) for 5 min at 25°C. The OD was read at 480/530 nm using the aforementioned microplate reader.

Measurement of apoptotic cells with TUNEL assay

A TUNEL assay was performed with an in situ cell-death detection kit according to the manufacturer’s instructions. Briefly, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After being washed with phosphate-buffered saline (PBS), the cells were permeabilized with 0.2% Triton-X 100 in methanol for 2 min at 4°C. The cells were then incubated with TUNEL assay solution at 37°C for 60 min. Finally, the cells were washed with PBS and mounted with fluorescent mounting medium. The number of TUNEL-positive cells was obtained by counting cells in 6 randomly selected microscopic fields from each coverslip with a ×20 objective. The percentage of TUNEL-positive cells in the total number of cells was calculated and averaged.
**Quantitative real-time PCR**

The miRNA samples were extracted using a commercial miRNA isolation kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer’s instructions. The miRNA reverse transcription was performed using miRCTe double-strand cDNA Synthesis kits (Tiangen, Beijing, China) according to the manufacturer’s instructions. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) of miR-409 was performed with a mirVana RT-qPCR miRNA Detection Kit (Ambion, Austin, USA), with each miRNA-specific primer. The miRNA level was presented as a level relative to U6 small RNA (taken as an internal control) using the $2^{-\Delta\Delta Ct}$ method.

**Western blot**

Total protein from the hippocampus was extracted with protein lysis buffer (20 mmol/L Tris-HCL, pH 7.6, 150 mmol/L NaCl, 1% NP-40) containing a protease inhibitor cocktail. Lysates were resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, USA). The blots were incubated with rabbit anti-APP (Abcam Inc., Cambridge, UK) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4°C. Then the blots were incubated with secondary antibodies conjugated with horseradish peroxidase (1:3,500 dilution, Santa Cruz Biotechnology, Santa Cruz, USA). The signals were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

**Statistical analysis**

The statistical analysis was carried out with SPSS v. 13.0 software for Windows (SPSS Inc., Chicago, USA). Values are expressed as means ± standard deviation (SD). Normal distribution of data was tested with the one-sample Kolmogorov–Smirnov test. A one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) test, were used to compare the measurement data of the 5 groups. Statistical significance was set at $p < 0.05$.

**Results**

**Effects of vitexin on learning and memory functions, cell apoptosis and expression of miR-409 in animal models**

To investigate the neuroprotective effect of vitexin on the hippocampus of rats, isoflurane anesthesia was used to induce brain damage, and then the effects on neuron apoptosis and memory function were investigated. Before exposure to the anesthetic, reference memory testing showed no differences between the groups in ELP (Fig. 1A).

but after exposure to isoflurane, the rats took significantly longer to reach the platform during the reversal test ($p < 0.05$). In addition, rats treated with 1 μM vitexin showed no decrease in the ELP. No differences were found between the isoflurane and 1 μM vitexin + isoflurane groups. However, the 10 μM and 100 μM vitexin groups showed significant decreases in the ELP ($p < 0.05$) and the effect was dose-dependent (Fig. 1B). Moreover, 3 μM and 10 μM vitexin could significantly decrease the apoptosis of hippocampal neurons and increase the expression of miR-409 ($p < 0.05$) (Fig. 1C,D). These results indicated that vitexin could protect hippocampal neurons from damage induced by isoflurane by regulating miR-409 expression.

**The effects of vitexin on cell viability, cell apoptosis, inflammatory cytokine expression, oxidative stress, and miR-409 expression in PC-12 cells**

To further study the protective mechanisms of vitexin on neurons, cell viability, the apoptosis and expression of inflammatory factors in PC-12 cells were investigated. As shown in Fig. 2A, isoflurane could significantly decrease the cell viability of PC-12 compared with the control group ($p < 0.05$), while this effect could be reversed by 1/100 μM vitexin. The apoptosis of PC-12 was significantly increased in the isoflurane group compared with the control group ($p < 0.05$), while this effect was also reversed by 100 μM vitexin. Expression levels of TNF-α, IL-6 and ROS showed the same trends with regard to the apoptosis of PC-12 cells (Fig. 2C,D,G). Levels of GSH, SOD (Fig. 2E,F) and miR-409 expression showed the same trends with regard to PC-12 cell viability (Fig. 2H). These results were consistent with those obtained in the animal experiments.

**The effects of miR-409 inhibitor on cell viability, cell apoptosis, inflammatory cytokines expression, and oxidative stress in PC-12 cells**

To evaluate the effect of miR-409 on isoflurane-induced neuron damage, cell viability, apoptosis, expression of inflammatory cytokines, and oxidative stress factors were assessed in PC-12 after transfection with miR-409 inhibitor. As shown in Fig. 3A, the expression level of miR-409 in the miR-409 inhibitor group was significantly decreased in comparison with the control group. As shown in Fig. 3B–H, knockdown miR-409 reversed the neuroprotective effects of vitexin. Cell viability and the levels of GSH and SOD in the vitexin + isoflurane + miR-409 inhibitor group were significantly increased compared with the vitexin + isoflurane + NC group ($p < 0.05$). Meanwhile, cell apoptosis and the expression levels of TNF-α, IL-6 and ROS in the vitexin + isoflurane + miR-409 inhibitor group were significantly increased compared with the vitexin + isoflurane + NC group ($p < 0.05$).
The effects of miR-409 mimic on cell viability, cell apoptosis, inflammatory cytokines expression, and oxidative stress in PC-12 cells

Following the investigations described above, cell viability, apoptosis and the expression levels of inflammatory cytokines and oxidative stress factors in PC-12 cells were measured after transfection with miR-409 mimic. As shown in Fig. 4A, the expression level of miR-409 in the miR-409 mimic group was significantly increased compared with the control group. As shown in Fig. 4B–H, overexpression of miR-409 showed the opposite trend, with miR-409 knockdown in PC-12 cells, which enhanced the neuroprotective effect of vitexin. Cell viability and the levels of GSH and SOD in the vitexin + isoflurane + miR-409 mimic group were significantly increased compared with the vitexin + isoflurane + NC group (p < 0.05). Meanwhile, cell apoptosis and the expression levels of TNF-α, IL-6 and ROS in the vitexin + isoflurane + miR-409 mimic group were significantly decreased compared with the vitexin + isoflurane + NC group (p < 0.05).

The effects of miR-409 on the AMPK/GSK3β pathway in PC-12 cells

It has been reported that the AMPK signaling pathway plays an important role in nerve protection. Therefore, this study assessed the expression levels of AMPK pathway-related proteins. The results showed that isoflurane could significantly decrease the expression levels of p-AMPK and p-GSK3β, while vitexin could significantly increase the expression levels of those proteins – an effect which was finally reversed by miR-409 inhibitor (Fig. 5A,B, p < 0.05).
However, compared with miR-409 inhibitor, miR-409 mimic showed the opposite trend, enhancing the effect of vitexin on the AMPK pathway (Fig. 5A,B). These results indicated that vitexin protected neurons from isoflurane-induced cell damage by upregulating the expression of miR-409, thus activating the AMPK signaling pathway.

**Discussion**

Previous studies have shown that isoflurane is potentially neurotoxic, inducing dose- and time-dependent damage to the nervous system (i.e., hippocampal slices, primary cortical and striatal neurons and neurosecretory PC-12 cells).\(^1\) Previous work in animal models had shown that isoflurane could induce neuronal apoptosis throughout brain, including the hippocampus and cerebral cortex.\(^{31,32}\) The present study showed that isoflurane could significantly increase apoptosis in the hippocampus neuron. It has also been reported that isoflurane can regulate the central cholinergic system, such as cholinergic receptor insensitivity and affinity, especially in the hippocampus,\(^{33}\) which is related to spatial learning and memory impairment.\(^{33,34}\) Our results support other findings which demonstrated that isoflurane exposure could impair learning and memory abilities in rats.\(^{35}\) Vitexin has been shown to reverse scopolamine-induced memory impairment at 100 μM.\(^{36}\) Our study, consistently
with Chen et al.,\textsuperscript{18} showed that 10/100 μM vitexin significantly increased the cell viability and decreased the apoptosis of PC-12, and reduced the expression levels of TNF-α, IL-6 and ROS in isoflurane-treated PC-12 cells. Oxidative stress increases pro-inflammatory gene expression, which triggers overproduction of ROS and results in a vicious cycle, provoking the occurrence and development of various diseases including nerve cell injury.\textsuperscript{37,38} Yang et al. suggested that vitexin protects PC-12 cells against 20 h of reoxygenation-induced injury by reducing the expression of ROS.\textsuperscript{36} In an animal model, Dong et al. found that vitexin protects against myocardial ischemia/reperfusion injury by inhibiting the inflammatory response.\textsuperscript{37} Furthermore, Min et al. demonstrated that vitexin can reduce hypoxia-ischemia neuronal brain injury.\textsuperscript{38} In addition, we found that vitexin could increase the expression of miR-409.

Increasing evidence indicates that the majority of miRNAs are expressed in the central nervous system\textsuperscript{42} and play important roles in brain development and nervous system diseases.\textsuperscript{43–45} Hence, miRNAs might participate in isoflurane-induced learning and memory impairment.

Fig. 3. The effects of miR-409 inhibitor on cell viability, apoptosis, inflammatory response, and oxidative stress in PC-12 cells after anesthesia. A. Expression of miR-409 detected using RT-PCR. B. Cell viability detected using MTT. C. Apoptosis of hippocampus neurons detected using TUNEL assay; apoptotic factors were detected using western blotting. D–G. The concentrations of TNF-α, IL-6, GSH, and SOD detected using ELISA kits. H. The concentration of ROS detected using ROS measurement. Error bars represent mean ± SD. Compared with the respective controls, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with isoflurane, *p < 0.05; compared with the vitexin + isoflurane + inhibitor NC group, &p < 0.05.
Yan et al. observed that isoflurane induces cytotoxicity and neuronal cell death by downregulating miR-214. Using a rat pup model, Luo et al. demonstrated that let-7d miRNA plays an important role in isoflurane-induced learning and memory impairment. In the present study, overexpression of miR-409 could decrease the apoptosis of PC-12 induced by isoflurane. Simultaneously, miR-409 overexpression reduced the inflammatory response and oxidative stress. It has been reported that ROS is involved in isoflurane-induced neurotoxicity, and increasing ROS increases the neurotoxic effect of isoflurane. The present study showed that isoflurane increased the expression of ROS, and that this was reversed by overexpression of miR-409. These results indicated that miR-409 could reduce isoflurane-induced neurotoxicity.

There is increasing evidence suggesting that the MAPK family, such as ERK1/2 and JNK, may be involved in the signaling of neuronal survival, regeneration and death. ERK1/2 is generally associated with pro-survival signaling that can activate CAMP-response-element-binding protein and upregulation of Bcl-2. c-Jun N-terminal kinases, on the other hand, participates in the apoptotic signaling.
In the present study, we found that vitexin could activate the AMPK/GSK3β pathway by upregulating miR-409, thus protecting neurons against isoflurane-induced injury. AMPK, as a key energy sensor of cellular metabolism, involves in metabolic stress, such as neurodegeneration, inflammation and oxidative stress. It has been reported that *Akebiae caulis* extract can inhibit oxidative stress through the AMPK/GSK3β pathway. Su reported that xanthohumol protects against LPS-induced acute lung injury, oxidative stress and inflammation damage by activating the AMPK/GSK3β signaling pathway. Wang et al. demonstrated that esculetoside A protects the liver against acetaminophen toxicity through the AMPK/GSK3β pathway. Furthermore, in the mature brain, post-mitotic neurons utilize MAP kinase and PI3K cascades in the regulation of key functions, such as synaptic plasticity and memory formation. These findings suggested that vitexin protects hippocampus neurons against isoflurane-induced oxidative stress and inflammation damage by activating the AMPK/GSK3β pathway in rats.

**Conclusions**

In conclusion, isoflurane impaired hippocampus-dependent learning and memory in rats, and 10/100 μM vitexin could significantly reduce the isoflurane-induced injury by upregulating the expression of miR-409 to activate the AMPK/GSK3β pathway. These results suggest that vitexin might be a promising candidate in neurotoxicity drug treatment. However, further studies are required.

