Isoflurane provides neuroprotection in neonatal hypoxic ischemic brain injury by suppressing apoptosis

De-An Zhao*, Ling-Yun Bi, Qian Huang, Fang-Min Zhang, Zi-Ming Han

The First Affiliated Hospital of Xinxiang Medical University, Department of Pediatrics, Weihui, China

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Abstract
Background and objectives: Isoflurane is halogenated volatile ether used for inhalational anesthesia. It is widely used in clinics as an inhalational anesthetic. Neonatal hypoxic ischemia injury ensues in the immature brain that results in delayed cell death via excitotoxicity and oxidative stress. Isoflurane has shown neuroprotective properties that make a beneficial basis of using isoflurane in both cell culture and animal models, including various models of brain injury. We aimed to determine the neuroprotective effect of isoflurane on hypoxic brain injury and elucidated the underlying mechanism.

Methods: A hippocampal slice, in artificial cerebrospinal fluid with glucose and oxygen deprivation, was used as an in vitro model for brain hypoxia. The orthodromic population spike and hypoxic injury potential were recorded in the CA1 and CA3 regions. Amino acid neurotransmitters concentration in perfusion solution of hippocampal slices was measured.

Results: Isoflurane treatment caused delayed elimination of population spike and improved the recovery of population spike; decreased frequency of hypoxic injury potential, postponed the onset of hypoxic injury potential and increased the duration of hypoxic injury potential. Isoflurane treatment also decreased the hypoxia-induced release of amino acid neurotransmitters such as aspartate, glutamate and glycine induced by hypoxia, but the levels of γ-aminobutyric acid were elevated. Morphological studies showed that isoflurane treatment attenuated edema of pyramid neurons in the CA1 region. It also reduced apoptosis as evident by lowered expression of caspase-3 and PARP genes.

Conclusions: Isoflurane showed a neuro-protective effect on hippocampal neuron injury induced by hypoxia through suppression of apoptosis.

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* Corresponding author.
E-mail: zhaodean17@gmail.com (D-A. Zhao).

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Introduction

Hypoxic brain injuries cause several pathologic conditions, which may also occur during neuro- and cardiac-surgeries and anesthesia. The mechanism underlying such hypoxic brain injury is still unclear. How to protect the brain from hypoxic injury and how to treat hypoxic brain injury remains clinically challenging. Hypothermia and pre-ischemia treatments have been shown protective effect on the brain, yet difficult to implement clinically, while pharmacological treatment clinically more practical. During the recent three decades, the neuroprotective effect of anesthetic drugs has drawn high attention from clinicians.

Isoflurane [2-chloro-2-[difluoromethoxy]-1; 1.1-trifluoroethane, CHF2-O-CHCI-CF3] is a halogenated ether used for inhalational anesthesia. Together with enflurane and halothane, it replaced the flammable ethers used in the pioneer days of surgery. Its name comes from being a structural isomer of enflurane, hence they have the same empirical formula. It is a racemic mixture of (R) and (S) optical isomers. Its use in human medicine is now starting to decline, being replaced with sevoflurane, desflurane, and the intravenous anesthetic propofol. Isoflurane is still frequently used for veterinary anesthesia. Propofol could reduce arterial blood flow in brain, intracranial pressure, and metabolism maintaining blood supply and oxygen ratio. It improved the oxygen supply during hypoxia suggesting protective effects of propofol against hypoxic brain damage.

Studies suggest that propofol plays a role in central nervous system (CNS) protection through the modulation of Ca2+, oxygen free radicals, γ-aminobutyric acid (GABA) receptor and N-methyl-D-aspartate (NMDA) receptor. Yet some data suggest that propofol had no brain protective effect after cardiac surgery and even worsened brain hypoxia and that hypothermia is neuroprotective rather than propofol. The volatile anesthetics all differ in potency, adverse effects, and cost, and are used extensively during surgery in human neonates and during neonatal animal research. Isoflurane was reviewed to have neuroprotective functions in studies with neonatal hypoxic ischemic brain injury. Isoflurane has been studied in animal models of various diseases, such as lipopolysaccharide (LPS)-induced acute inflammation of the lung, acute lung injury, glucose-induced oxidative stress, renal ischemia/reperfusion injury, and cardiac injury. Isoflurane was shown to provide protection from injury and improve various negative functional outcomes in these models.

Neonatal hypoxia ischemia is a major cause of mortality and neurologic deficits such as cerebral palsy, mental retardation, and epilepsy in the perinatal period. Several pathophysiological factors have been implicated in the hypoxia ischemia, including inflammatory mediators, excitotoxicity, and oxidative stress. The use of isoflurane as...
an anesthetic in the surgery for modeling neonatal hypoxia ischemia has led to speculations that isoflurane may be having protective effects on neonates. In particular, isoflurane shows neuroprotection in several stroke models including subarachnoid hemorrhage (SAH),19 middle cerebral artery occlusion (MCAO),20,21 intracerebral hemorrhage,22 traumatic brain injury,23 and neonatal hypoxic ischemic brain injury.24 A study showed that 2 h exposure to isoflurane did not cause neuroapoptosis in adult brains and suggests that delayed reduction in astroglial processes after isoflurane exposure may explain why some volatile anesthetics can confer neuroprotection after experimental stroke.25

Isoflurane has been claimed to be both neuroprotective and neurotoxic yet the claim about isoflurane causing neural apoptosis remains controversial. In this study, we investigated the effects of isoflurane exposures on hippocampus slices of rat brain and assessed the apoptotic function. To investigate the effects and mechanisms of isoflurane, neurological changes, degrees of neurogenesis, and apoptosis were studied in rats after exposure to various concentrations of isoflurane. The overall goal of this study is to find evidences demonstrating that isoflurane treatment has neuroprotective effects on brain during hypoxia. Similar to the results in several studies, we found no evidence of brain cell death or neurogenesis in neonatal hypoxic ischemic brain tissues after isoflurane anesthesia.

Methods

Experimental animals

All the animal protocols were approved by the Institutional Animal Ethics Committee. Animals were housed and maintained under defined protocol with a 12 h light–dark cycle and the room temperature was maintained at 25 ± 1 °C. Sixty Sprague–Dawley (SD) rats at postnatal day (P) 7 (weight, 16–18 g) were randomly divided into five groups (n = 12). Group (i) control reviewed normoxia treatment. Forty eight SD rats were subjected to hypoxia then distributed in further four groups: (ii) hypoxia (Hx); (iii) Hx-isoflurane 0.5% treated; (iv) Hx-isoflurane 1.5% treated; (v) Hx-isoflurane 2.5% treated.

Neonatal hypoxia–ischemia

Rats of groups (ii–v) were anesthetized using 3% halothane followed by a 3 mm midline cervical incision as per standard procedure. The left carotid artery was exposed and coagulated using a bipolar electrocauterity unit followed by closure of the incision using topical surgical adhesive. After a 3 h recovery period, the mice were then placed in a chamber containing 8% O2/92% N2 for 45 min. The temperature in the chamber was maintained at 37 °C. Control mice (zero time) were anesthetized and underwent exploration of left carotid artery without cauterization.

Isoflurane exposure

Rats were prepared for isoflurane exposure 18 h after hypoxia. Rats were placed in plastic containers and exposed to isoflurane for 6 h continuously using air as a carrier with a total gas flow of 2 L/min. During the isoflurane exposure, containers were maintained to 37 °C using a heating device. The levels of isoflurane, oxygen and carbon dioxide were monitored in the chamber using a gas monitor. After 6 h, isoflurane administration was terminated and the rats were exposed to air solely. After observing free movement in rats, they were placed back into the maternal cage. The respiratory frequency and skin color of the rats were monitored during isoflurane exposure. Rats were observed for any case of apnea or hypoxia and were immediately exposed to air and excluded from the experiment. Rats in the control group were placed into similar container as the rats in the isoflurane group, but were exposed to air alone for 6 h.

Tissue preparation

Rats were anesthetized with isoflurane after the recovery period. A transcardial perfusion was performed with buffered 10% formalin phosphate according to previous protocol. The brains were extracted from the skulls through surgical procedure. Brains were stored in buffered 10% formalin for 48 h. Then the tissue was frozen-sectioned into 30 mm slices using a Leica SM2000R microtome (Leica, Germany). Sections from the hippocampal region were randomly selected for histological and other studies.

Histopathological examination

The rats exposed to isoflurane as well as control group were sacrificed at 6 h (n = 3). Hippocampal slices from each group were fixed in 0.1% methanol and embedded in wax after dehydration. Tissue blocks were sliced in 5 mm thick sections and stained with hematoxylin and eosin (H&E) as per standard protocol. The results were examined in detail under a light microscope so as to determine morphological changes in parts of the CA1 and CA3 regions.

Extracellular potential recording

Electric probes were inserted into Schaffer lateral fiber at CA3 and extracellular potential was recorded as described earlier. Stimuli (0.6 mA, 0.1 Hz, 100 ms) were given with a 10-s interval. Evoked potentials including orthodromic population spike (OPS) and hypoxia injury potential (HIP) were recorded at CA1. Only brain slices that had stable OPS (43 mV) for at least 20 min were selected for experiments. Hypoxic groups were incubated in ASCF without glucose and oxygen (ASCFOGD) for 15 min followed by normal ASCF perfusion. In hypoxic and treatment groups, every OPS was recorded before its disappearance. Each group contained eight samples. OPS decay is defined as the time period when OPS become undetectable after oxygen deprivation. The amplitude of OPS recovery is defined as the ratio of OPS amplitude after 1 h of normal ASCF perfusion over OPS amplitude before oxygen deprivation. The ratio of OPS recovery is defined as the percentage of hippocampal slices in which OPS amplitude recovery reach 60% of OPS amplitude before oxygen deprivation. HIP onset is defined
as the time period when HIP is recorded after oxygen deprivation. HIP duration is defined as how long HIP lasts. HIP incidence is defined as the percentage of hippocampal slices in which HIP were recorded after oxygen deprivation.

High performance liquid chromatographic (HPLC) analysis

Rat brain hippocampal slices from each group (n = 3) were incubated in ASCF (NaCl 124 mM; KCl 3.3 mM; NaH2PO4 1.24 mM; MgSO4 2.4 mM; NaHCO3 25.7 mM; CaCl2 2.4 mM) with and without glucose (10.0 mM) at 37.5 °C for 2–2.3 h. The flowing volume was 200 mL/min/L. The ASCF from 15 min of perfusion from each group was collected and centrifuged to clear supernatant. Supernatant was processed for HPLC analysis as per standard protocol with necessary modifications. The mobile phase A was Sodium Acetate (0.5 M, pH 6.0) with 0.05% THF. The mobile phase B was methanol. The following washing gradients (minutes, Phase B %) were used: 0, 30%; 7, 60%; and 9, 30%; with velocity of 0.9 mL/min, temperature at 35 °C, lem at 330 nm and lex at 456 nm. Derivation agent used was OPA, 2-MCE, Boric acid–sodium hydroxide and methanol (Ph 9.6). Derivation agent (25 mL) and sample (25 mL) was incubated for 2 min at room temperature. Injection volume was 20 mL. Standard curves were prepared using 10; 5; 2.5 and 0.625 mM of Asp, Glu, Gln, Gly or GABA (HPLC grade). The concentrations (mM) of Asp, Glu, Gln, Gly and GABA in perfusion ASCF were calculated based on standard curves.

Real time-PCR (RT-PCR)

Hippocampal slices from each group (n = 3) were snap frozen at −80 °C in RNasefree microfuge tubes at the moment of dissection for RT-PCR. Total RNA was isolated from tissues using Trizol reagent (Invitrogen, USA) according to manufacturer’s instructions. Total RNA (1 µg) was incubated with 200 ng random hexamers, 0.5 mM dNTPs, and RNase-free water at 65 °C for 10 min (all from Invitrogen). Then, 5× first strand buffer, 5 mM DTT, 40 U RNase A, and 200 U reverse Transcriptase (SuperScript III) was added. The reaction mixture was incubated at room temperature for 5 min followed by at 50 °C for 60 min, at 70 °C for 15 min, and at 4 °C for 10 min. Real-time quantitative PCR was performed using an ABI 7500 Real Time PCR System (Applied Biosystems). Assays for target cDNA levels were performed using TaqMan MGB probes labeled with FAM dye (Applied Biosystems) in a 20 µL reaction containing 2 µL of cDNA, ROX passive reference dyes and TaqMan Universal PCR mix (Applied Biosystems). The specific primer sets used and their part number are: Parp1 poly (ADP-ribose) polymerase 1 25591 Rn00565018_mL and Casp3 caspase 3, apoptosis related cysteine protease 25402 Rn00563902_mL. Relative quantities of both mRNAs were established by normalizing their levels to that of 18S in the same cDNA.

Statistical analysis

All values are expressed as mean with standard deviations (SD). Data were analyzed using the SPSS v.17 and Systat SigmaPlot 10.0 statistical software. Statistical significance was calculated by Student’s t test and one way analysis of variance (ANOVA) for multiple comparisons. p-Values < 0.05 were considered statistically significant.

Results

Effect of isoflurane treatment on OPS and HIP after neonatal–ischemia hypoxia

OPS and HIP were recorded at the CA1 region. In the hypoxia group, the average decay time of OPS was 144 ± 21 s; the ratio of OPS recovery was 22% and the amplitude of recovery was 24 ± 9%. In 1.5 and 2.5% isoflurane exposure groups, the decay time of OPS was significantly prolonged and the ratio of OPS recovery and the amplitude of recovery were increased as compared with that of hypoxia group. No significant changes of the decay time, recovery ratio and amplitude of OPS were found in 0.5% isoflurane exposure group (Fig. 1A). In the hypoxic group, the average onset time of HIP was 403 ± 56 s; the average duration of HIP was 104 ± 14 s and the incidence of HIP is approximate 100%. The incidence of HIP was reduced; the onset of HIP was postponed and the duration of HIP was extended in 1.5 and 2.5% isoflurane exposure groups as compared with that of hypoxic group. The above parameters were statistically not significant in 0.5% isoflurane exposure group (Fig. 1B).

Effect of isoflurane exposure on release of amino acid neurotransmitters

Amino acid neurotransmitters like such as aspartate (Asp), glutamate (Glu), glutamine (Gln), glycine (Gly) and GABA are commonly circulated and have important functions in CNS. The high concentration of excitatory amino acids (EAA) in synaptic cleft and extracellular fluid during hypoxia is a common pathway causing excitatory toxicity and neuronal deaths. Most amino acid neurotransmitter assays measure the total amount of amino acids in specific brain areas or nuclei, which may also contain amino acids from protein metabolism. Thus, the total amino acid amount of the hippocampus may not reflect the amount of amino acids as a neurotransmitter. In this study, we directly measure the concentration of amino acid neurotransmitters in perfusion solution of hippocampal slices during hypoxia using HPLC. The concentrations of neurotransmitters Asp, Glu, Gly and GABA were significantly (p < 0.05) increased in the hypoxic group as compared with that of normoxia control group (Table 1). The concentrations of Asp, Glu, Gly and GABA were increased by 1.32; 1.27; 1.78; 1.17 folds, respectively. Isoflurane exposure reduced the levels of Asp, Glu and Gly in dose-dependent manner especially at 1.5% and 2.5%. However, isoflurane exposure continuously increased the levels of GABA release with statistical significance. The level of Gln release was altered neither by hypoxia nor by isoflurane exposure (Table 1).
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Figure 1  The effect of isoflurane on OPS and HIP. Decay time (s), recovery rate (%) and amplitude recovery (%) for OPS (A); onset (s), duration (s) and incidence (%) for HIP (B) were estimated as in Materials and Methods (n = 3; * p < 0.05 vs. hypoxic group).

Isoflurane improves morphological changes in hypoxic hippocampal slice

The histological studies using HE staining (Fig. 2) showed that pyramid neurons in the CA1 region of the hippocampus became swollen. The boundaries of cells become blurred and the nuclei were dark and shrunken. HE data suggests that cell damage occurred in the hypoxic group. Furthermore, isoflurane treatment ameliorated those morphologic changes of pyramid neurons induced by hypoxia in dose-dependent manner.

Isoflurane suppressed neonatal hypoxia ischemia induced apoptosis

We quantitated levels of caspase-3 and PARP mRNA from the hippocampus using RT-PCR. Relative quantities of both mRNAs were established by normalizing their levels to that of 18S in the same cDNA. The log expression levels of genes were expressed in terms of fold-change compared to control animals. Fig. 3 shows that caspase-3 mRNA was elevated about 2-fold after neonatal hypoxia ischemia as compared with normoxia control. Increases in caspase-3 mRNA is a

<table>
<thead>
<tr>
<th>Group</th>
<th>Asp ± 0.12</th>
<th>Glu ± 0.12</th>
<th>Gln ± 0.05</th>
<th>Gly ± 0.07</th>
<th>GABA ± 0.08</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.03 ± 0.12</td>
<td>1.85 ± 0.12</td>
<td>0.77 ± 0.05</td>
<td>1.05 ± 0.07</td>
<td>1.79 ± 0.08</td>
</tr>
<tr>
<td>Hypoxia (Hx)</td>
<td>2.68 ± 0.14</td>
<td>2.35 ± 0.09</td>
<td>0.76 ± 0.05</td>
<td>1.87 ± 0.04</td>
<td>2.09 ± 0.06</td>
</tr>
<tr>
<td>Hx-ISF 0.5%</td>
<td>2.61 ± 0.15</td>
<td>2.42 ± 0.11</td>
<td>0.75 ± 0.04</td>
<td>1.78 ± 0.07</td>
<td>2.21 ± 0.09</td>
</tr>
<tr>
<td>Hx-ISF 1.5%</td>
<td>2.44 ± 0.13</td>
<td>2.01 ± 0.14</td>
<td>0.71 ± 0.05</td>
<td>1.65 ± 0.09</td>
<td>2.43 ± 0.12</td>
</tr>
<tr>
<td>Hx-ISF 2.5%</td>
<td>2.41 ± 0.14</td>
<td>1.92 ± 0.12</td>
<td>0.72 ± 0.04</td>
<td>1.57 ± 0.07</td>
<td>2.55 ± 0.14</td>
</tr>
</tbody>
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ISF, isoflurane.

a p < 0.05 vs. the normoxia control group.

b p < 0.05 vs. the hypoxic group.
Figure 2  HE staining of hippocampal slices. (A) normoxia control; (B) hypoxia (Hx); (C) Hx-isoflurane 0.5%; (D) Hx-isoflurane 1.5%; (E) Hx-isoflurane 2.5%. HE staining was performed as described in Material and Methods ($n = 3$).

direct evidence of apoptosis induction in hippocampal slices. Apoptosis induction requires activation of caspase-3, which cleaves nuclear substrate PARP. Analysis of PARP mRNA shows that its level increased 1.86-fold after hypoxia as compared to normoxia control. Increases in PARP mRNA confirmed the apoptosis induction in hippocampal slices. Furthermore, isoflurane exposure to these tissues showed dose-dependent decrease in the expression levels of both genes. Isoflurane decreased the levels of caspase-3 and PARP mRNAs with statistical significance. These results show that apoptosis induced by neonatal hypoxia ischemia is suppressed by isoflurane exposure.

Discussion

This study investigated the effects of the volatile anesthetic isoflurane on possible mechanisms of anesthetic-induced neuroprotection and neurotoxicity in hippocampus of rat brain under hypoxia ischemia. We mainly observed that isoflurane does not alter neurogenesis, nor does it cause neuronal cell death, in various formations of the hippocampus. Our findings demonstrate that isoflurane causes neuroprotection by suppressing apoptosis induced by hypoxia.

The hippocampal slice has been commonly used in hypoxia-induced brain injury related research and for their pharmacologic protection. The pyramid neurons in the hippocampus are the most sensitive cells to hypoxia, of which, the pyramid neurons in CA1 are more vulnerable to hypoxia. Because synapses is formed between CA1 and CA3, the orthodromic population spike (OPS) can be recorded in CA1 when Schaffer collateral in CA3. Thus the OPS demonstrate apparent changes during hypoxia. Previous studies have shown a reduction of OPS amplitude during hypoxia, an indicator of synaptic dysfunction and loss of excitatory ability. Such OPS changes are reversible if oxygen supply is restored in time and the delay of OPS disappearance suggests that the neurons are less vulnerable to hypoxia. Thus,
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Figure 3  Quantitative real time-PCR for caspase-3 (A) and PARP (B). Total RNA was isolated from hippocampal slices (n = 3). The cDNA was synthesized and qRT-PCR was performed as described in Materials and Methods.

The decay time of OPS after hypoxia can be used as a parameter to evaluate tolerance of neurons. The hypoxic injury potential (HIP) is an indicator of the irreversible hypoxic damage induced in hippocampal slices. The onset and duration of HIP is correlated with hypoxic damage. The longer HIP appearance correlates with easier recovery for hippocampal slice after restoration of oxygen supply. If onset of HIP is postponed or extended, neuron damage will be delayed.

We investigated how isoflurane affects the evoked potentials of hypoxic hippocampal slice. In hypoxic groups, the average decay time of OPS was about 144 s, the recovery ratio of OPS and amplitude of OPS were both small after restoration of oxygen and glucose supply (Fig. 1A). It indicated the hypoxic damage of synaptic transmission between hippocampal neurons. The exposure to isoflurane caused dose-dependent increase in recovery ratio of OPS and amplitude of OPS. It suggests that isoflurane treatment increased the hypoxic tolerance of the hippocampal slice. The delay of HIP onset, the extension of HIP duration and decreased incidence of HIP in different dosage of isoflurane also demonstrated that isoflurane exposure postpones the occurrence of irreversible hypoxic damage and increases duration of reversible damage. Collectively, it suggests that isoflurane treatment reduces the hypoxic damage of neurons and protects the synaptic functions from hypoxic damage.

The protective effect of isoflurane may result from inhibition of neurotransmitter amino acid like Glu, a major neurotransmitter in the CA1 region of the hippocampus. Reports suggest that excitatory amino acids (EAA), including Glu and Asp, play a key role in the hypoxic brain damage mechanism. NMDA receptor is a major receptor of EAA and its activation results in intracellular Ca²⁺ overload in hypoxia. In hypoxia group, Asp and Glu concentrations in perfusion solution were increased 1.32 and 1.27-fold, respectively (Table 1). Isoflurane treatment reduced the hypoxia-induced release of Asp and Glu, thus reducing the excitatory toxicity. GABA is an inhibitory neurotransmitter in CNS that can protect neurons from hypoxic damage. This study showed that isoflurane treatment can increase the concentration of GABA in perfusion fluid, which may be a neuroprotecting mechanism of isoflurane during hypoxia. Another anesthetic propofol was reported to increases affinity between GABA and its receptor, thus activating the GABA receptor directly to result in enhanced membrane current flow and GABA-induced hypopolarized inhibition. Glycine (Gly) is an EAA modulator that increases the sensitivity of NMDA receptor to EAA, amplifying the excitatory toxicity induced by EAA. Gly was found increased in the hypoxia group, which may facilitate the excitatory toxicity (Table 1). Isoflurane treatment reversed the elevation of hypoxia-induced Gly concentration in dose-dependent manner, suggesting the CNS protecting effects of isoflurane. The data showed none effect of isoflurane on the release of Gln, which may not directly associated with the neuroprotective mechanism of isoflurane. Brain hypoxia may cause ATP exhaustion, influx of Na⁺ and Cl⁻ efflux of K⁺ and H₂O resulting in cellular edema. In contrast, excessive release of Glu over-excites the neurons and causes an influx of Ca²⁺, and increase in NMDA dependent Ca²⁺ influx. This results in overload of intracellular Ca²⁺ that initiates enzyme activation, protein hydrolysis, formation of oxygen free radicals, DNA damage and neuronal cell death. Our data shows that isoflurane diminishes hypoxia-induced cellular edema and mitochondrial damage. Based on data, we hypothesize that the other potential mechanisms by which isoflurane protects CNS from hypoxic damage include modulation of Ca²⁺, clearance of oxygen free radicals, up-regulation of GABA receptor and inhibition of NMDA receptor. Furthermore, we found that isoflurane treatment caused inhibition of hypoxia induced levels of mRNA of caspase-3 and PARP. Apoptosis can be executed mainly by two pathways: caspase-9 dependent mitochondrial intrinsic and caspase-8 dependent extrinsic. Both pathways lead to activation of executioner caspase-3 which ultimately causes cell death by cleaving a number of cytoplasmic and nuclear substrates like PARP. This study found that isoflurane suppressed the expression levels of apoptotic genes caspase-3 and PARP in dose-dependent manner (Fig. 3).

In conclusion, isoflurane exposure provides neuroprotection against to hypoxic ischemic brain injuries in rat brain. Isoflurane exposure reduces the hypoxic damage of neurons and protects the synaptic functions from hypoxic damage. Isoflurane reduces the levels of hypoxia-induced release of Asp and Glu and enhances the release of GABA. These amino acid neurotransmitter help protect CNS from...
hypoxic damage. The basic mechanism behind neuroprotection appears to be inhibition of apoptosis induced by hypoxia. This study shows insights for using isoflurane as a safe anesthetic in clinical practices as well as in veterinary practices.

Conflicts of interest

The authors declare no conflicts of interest.

References


