Rhodioloside inhibits apoptosis of hippocampal neurons exposed to sevoflurane via cAMP/PKA signaling pathway

Yuqiang Su, Yan Bai, Zhonglei Zheng, Xiaoying Fan*
The Second Affiliated Hospital of Xi'an Medical College, Department of Surgical Anesthesia, Xi'an, 710038, China

*For correspondence: Email: dced7848@126.com; Tel: +8619654321022

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Abstract

Purpose: Neural injury affects patients after using inhalational anesthetics such as sevoflurane. Rhodioloside, a compound which is obtained from the Rhodiola rosea plant has been implicated to be the most commonly used psychostimulant that can improve a range of conditions. The study was aimed at finding the molecular mechanism underlying the Rhodioloside treatment of sevoflurane-injured hippocampal neurons.

Methods: Main hippocampal neurons, secluded from Sprague Dawley embryonic rats were employed to create an injury model using 3 % sevoflurane. The sevoflurane-injured hippocampal neurons were treated with varying concentrations (10, 20, 40 and 80 µM/ml) of Rhodioloside to create different experimental groups: RHSD10+SEV, RHSD20+SEV, RHSD40+SEV, RHSD80+SEV, while untreated cells were considered as the Control group. Cell viability was identified using the CCK-8 assay. The CFSE assay was used to verify the promotion function of Rhodioloside on cell differentiation of neurons. FCM assay was employed to determine cell proliferation and apoptosis. Expression levels of apoptosis-related factors, like Caspase-3, Bcl-2 and Bax were examined by RT-qPCR, while Western blot was used to measure phosphorylation of PKA.

Results: Rhodioloside stimulated cell viability and prevented cell apoptosis in sevoflurane-injured hippocampal neurons in doses between 10-80 µM. The apoptosis-inhibitory effect of Rhodioloside was observed to be through cAMP/PKA pathway activation. Also, expression levels of Bcl-2, and PKA were enhanced and the level of Caspase-3 and Bax was reduced in a dose-dependent pattern. The PKA inhibitor reversed the above observation in the 40 µM Rhodioloside-treatment.

Conclusion: Rhodioloside promoted cell viability and prevented apoptosis of primary hippocampal neurons injured by sevoflurane, through cAMP/PKA pathway activation. Inhibition of PKA network deteriorated the function of Rhodioloside by stimulating cell apoptosis. Our findings present a novel evidence that Rhodioloside could attenuate neurotoxicity of inhalational anesthetics.

Keywords: Cell apoptosis, cAMP/PKA pathway, Hippocampal neurons, Rhodioloside, Sevoflurane

INTRODUCTION

Sevoflurane is among the most used anesthetics in cesarean delivery and pediatric clinical process due to its swift induction and recovery features. Studies have shown over the years that the use of anesthesia in youngsters below 3 years could impact their development and behavior, thereby leading to cognitive disorders [1]. The critical period in the toxicity of anesthesia...
is dependent on the fast synaptogenesis, which continues from mid gestation to several years after birth [2]. Exposure of sevoflurane on immature brain, which could lead to neurotoxicity is associated with alterations in behavior of children later in adulthood and memory, reading, writing and mathematical learning challenges [1,3-6]. More studies further indicated that sevoflurane has the ability to induce neural apoptosis of a developing brain of a child [7,8]. It is essential to find a solution to anesthetic neurotoxicity, given that sevoflurane is used frequently in childbirth and surgeries to alleviate pain.

Rhodiola, often referred to as salidroside is a compound found in the Rhodiola rosea (also known as golden root) plant and is one of the known potential compounds responsible for antidepressant and anxiolytic actions in plants [9]. The plant is a perennial herb in the family Crassulaceae and grows naturally in Artic Europe, including Britain, Asia and North America. It is abundantly and widely distributed in China. In Europe and Asia, R. rosea is mentioned as the most commonly used psychostimulant that can improve a range of conditions, including depression, anxiety, fatigue, anaemia, endurance, altitude sickness, insomnia and impotence [10,11]. A recent study found that the plant’s supplement strikingly improves aerobic exercise performance after short-term high altitude training and has lots of biological activities, such as anti-stress and anti-fatigue [12,13]. Rhodiola exerts protective effects on chronic intermittent hypoxia-induced mitochondrial-dependent apoptosis in cardiac cells [14]. Accumulating evidence indicates that the plant extracts protect against cerebral ischemia-reperfusion injury in rat brain [15]. Pharmacological studies suggest that Rhodiola plant promotes cognitive function and relieves brain fatigue, clears reactive oxygen species, reduces oxidative stress, enhances physical endurance, ameliorates metabolic dysfunction, boost immunity and exerts anti-tumor effects [16]. In recent years, Rhodiola has attracted increasing attention because of its cognitive protective effects, antioxidant effects, and ability to scavenge reactive oxygen species. The potent component rhodioloside has been found to increase activation of the hippocampus and promote cell proliferation and differentiation of the neurons. Besides, rhodioloside improves neurogenesis in the hippocampus, hence boost cognition through regulation of apoptosis of the hippocampal neurons, hence prevents mental and memory death in scopolamin-treated rats [17]. There is however no clarity on how rhodioloside protects neurons from injury caused by sevoflurane.

Apoptosis is referred to as a programmed cell death and works by eliminating dysfunctional cells to keep up ordinary functions of body tissue. Anomalous apoptosis can lead to cell damage or loss of life [7]. Many reports show that sevoflurane triggers the stimulation of cysteine aspartate-specific protease (Caspase) and the apoptotic pathway [18]. Adenosine 3’,5’ cyclic monophosphate (cAMP) and cAMP/PKA, are the cAMP-dependent protein kinase (PKA) [19]. Recent studies have confirmed that differential effects of cAMP may be contributed to the features of PKA signaling pathway and impact positively on the neuroprotective process [20]. The antiapoptotic effect of ODN on neural cells is mediated through activation of the ODN metabotropic receptor, positively coupled to PKA, PKC and MAPK/ERK transduction pathways, which ultimately reduces the pro-apoptotic gene, Bax and stimulates Bcl-2 expressions, and inhibits intracellular reactive oxygen species accumulation [21]. The sevoflurane neurotoxicity in not fully formed brain is associated with cAMP/PKA signaling pathway and other related factors leading to cell death, such as Caspace-3, B-cell lymphoma/leukemia-2 (Bcl-2) and Bcl-2 associated X (BaX). It is essential to understand the role of rhodioloside on sevoflurane-injured neurons via the cAMP/PKA pathway.

In this study, we therefore used the sevoflurane-injured rat hippocampal neuron and constructed its model to authenticate the likely role of rhodioloside on cell apoptosis triggered by sevoflurane. The role of cAMP/PKA signaling pathway was examined, which could be a potential clinical use of rhodioloside as a solution to anesthetic neurotoxicity.

EXPERIMENTAL

Primary nerve cell cultures

Matured female Sprague-Dawley rats, weighing 200 to 300 g, were bought from Beijing Vital River Company, Beijing, China and kept for two weeks under typical environment of 22-24 ºC, 55-65 % moisture in a 12-hour light/dark cycle. The process was carried out after breeding the female and male rats. The fetuses were then secluded for the study and an approval was obtained from the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Xi'an Medical College. The rats were sacrificed by cervical dislocation, wrapped up and sterilized using alcohol. After the sterilization of the uterus, the fetus was carefully removed.
The hippocampus soft tissues were secluded and broken down by 0.125 % trypsin for 30 min and nerve cells were gathered and cultured in Neural basal media, supplemented with 2 % B27, 10 mmol/l HEPEES, and 0.5 mmol/l L-glutamine in 5 % CO₂-containing incubator at 37 °C. Cell culture media were altered every three days. Cell morphology was studied using DM8 optical microscope after being cultured for 3 and 7 days, respectively.

After 7 days, the primary hippocampal neurons were pre-treated with Rhodioloside (RHSD) with varying concentrations (10, 20, 40 and 80 μM/ml) for six hours, and injured by 3 % sevoflurane gas assortment of 95 % oxygen and 5 % carbon dioxide; subsequently, the flow ratio was delivered at 2 l/min. An injury model was constructed using 3 % sevoflurane, a higher concentration than what is usually used in clinical application and scientific research. The experimental groups were termed: RHSD10+SEV, RHSD20+SEV, RHSD40+SEV, RHSD80+SEV, respectively. SEV group was given to cells that were treated with 3 % sevoflurane gas alone, RHSD40 for cells treated with 40 μM/ml RHSD, and untreated cells were considered as the Control group. Besides, to have the finest repair outcome, RHSD40+SEV cells were selected to be treated with 5 μmol/l ERK1/2 inhibitor PD98059 1 hr before 40 μg/ml RHSD treatment (cAMP/PKA inhibitor+RHSD40+SEV group). The changes were compared to that of the Control, SEV, RHSD40+SEV groups.

Cell counting kit-8 assay

Cell practicability of primary hippocampal neurons in the experimental groups (Control, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV, RHSD80+SEV, LBP80+SEV) were assessed using the CCK-8 assay in dedicated times (2, 4 and 6 hr), based on the manufacturer’s protocols. After the treatment, the cells were planted in 96-well plates and reacted with 20 μl CCK-8 reagent for 1 hr in 5 % CO₂-containing incubator at 37 °C. The ophthalmic density standards were measured using the microplate reader at 450nm.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) assay

Using the CFSE cell proliferation kit (Invitrogen, USA), the proliferation potential of cells in different clusters (Control, SEV, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV groups) was examined. The assay was performed based on the manufacturer’s instructions. Cells were re-suspended to 1x10⁶/ml in 1 ml preheated phosphate buffer solution (PBS) in sterile centrifuge tubes. An aliquot of 2 μl CFSE (5 mmol/l) stock reagent was added into cell suspension to a final concentration of 10 μmol/l. Cells were kept warm at 37°C for 10 min and then in 10 ml icy culture media for 5 min in the dark. Finally, cells were inoculated in 24-well plates (1x10⁵/well) and kept warm with 5 % CO₂ at 37°C, then cells were digested, collected and detected by FACS Calibur flow cytometer without light in the entire process.

Apoptosis detection

Apoptotic rates of hippocampal neurons in the experimental groups (Control, SEV, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV groups) were determined by Annexin V/PI (propidine iodide) double-stain assay, according to the company’s instructions (BioVision, Inc., Milpitas, CA, USA). Annexin V/FITC (fluorescein isothiocyanate) was used to measure the Phosphatidylserine exposed to the outside of cell membrane in early stage of apoptosis. PI also helped to detect DNA within membrane-injured cells in the late-stage of apoptosis. Cells were re-suspended by 100 μl binding buffer to a final concentration of 1x10⁶ cells/ml. Then, 5 μl Annexin V/FITC and 10 μl PI (20 μg/ml) were added. After incubation for 15 min in darkness at room temperature, a flow cytometer (BD Biosciences) was used to analyze cells, with 488 nm as thrilling light wavelength, and 515 nm (FITC), 560 nm (PI) as spotting light wavelengths.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was done to analyze mRNA expression levels of apoptosis-related factors such as Caspase-3, Bax, Bcl-2 in different groups, including Control, SEV, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV and cAMP/PKA inhibitor+ RHSD40+SEV groups. GAPDH was used as an inner reference. Initially, TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total RNA and reversely transcribed to cDNA with a Paramout Strand cDNA kit (Takara Bio, Inc., Otsu, Japan), based on the manufacturer’s instructions. PCR strengthening was carried out using the SYBR Premix Ex Taq kit (Takara Bio, Inc.). Briefly, after pre-denaturation at 95°C for 30 sec, amplification of 40 cycles were conducted: denaturation at 95°C for 5 sec, annealing/extension at 60°C for 30 sec, which was performed in ABI 7300 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were presented in Table 1.
Table 1: Nucleotide sequences of primers used for targeting genes

| Primer   | Direction | Nucleotide sequence                      |
|----------|-----------|-----------------------------------------|
| Caspase-3| Sense     | 5'-TGG AATTCAGCTCGCAATG -3'              |
|          | Antisense | 5'-CAGGG CGTTGTTCCAAAAA-3' 5'            |
| Bax      | Sense     | 5'-AGATCATGAAGAGACGG GGCCT-3' 5'         |
|          | Antisense | 5'-ATCCTTCAGCTGCAG-3'                    |
| Bcl-2    | Sense     | 5'-AACTCTTCAGGGATGG GG TG-3' 5'          |
|          | Antisense | 5'-GCTGGGGCATATAGT TCC A-3'             |
| GAPDH    | Sense     | 5'-AGTCTACTGGCGTCTTC ACC-3' 5'          |
|          | Antisense | 5'-CCACGATGCCAAAGTT GTCA-3'             |

Western blot analysis

Western blotting was conducted to determine protein levels, including apoptosis-interrelated factors (active-Caspase-3, Bax, Bcl-2) and p-PKA (phosphorylated PKA), t-PKA (total-PKA), in diverse clusters which include: Control, SEV, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV, RHSD40+SEV and PKA inhibitor+RHSD40+SEV groups. Proteins were first acquired and the concentration was determined by BCA assay (Beyotime Institute of Biotechnology, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then used to concentrate and separate the proteins, and electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). After the blockage with 5 % nonfat dry milk for an hour, blotted membranes were incubated with specific crucial antibodies overnight at 4 °C, respectively. GAPDH was used as loading control. The primary antibodies were as follows: anti-PKA (Abcam; ab17942, 1:1,000), anti-p-PKA (Cell Signaling Technology, Inc., Danvers, MA, USA; 4370, 1:2,000), anti-GAPDH (Abcam; ab9485, 1:2,500). After that, membranes were incubated with the appropriate secondary antibody goat anti-rabbit HRP-conjugated IgG H&L (Abcam; ab6721, 1:5,000) for 2 hr. The PVDF membrane were exposed to and detected by X-ray film with enhanced chemiluminescence (ECL) detection system reagents (Amersham; GE Healthcare, Chalfont, UK).

Statistical analysis

All the functional assays were repeated three times. All data were presented as mean ± standard deviation of three independent replicates. Statistical analysis was conducted by means of a SPSS 22.0 statistical package (IBM Corp., Armonk, NY, USA), and Welch's one-way analysis of variance (ANOVA) was used to analyze the dissimilarities among multi-groups. Both Games-Howell test was also employed. P<0.05 (***) and P<0.01 (****) were considered significant.

RESULTS

Rhodioloside promotes cell viability of hippocampal neurons injured by sevoflurane

Hippocampal primary neurons were isolated from SD embryonic rats. CCK-8 assay was used to detect the function of rhodioloside with different concentrations (10, 20, 40 and 80 μM/ml) on cell viability of hippocampal primary neurons treated with 3 % sevoflurane, at 6 hr. The function of 40 μM/ml RHSD alone was also detected. RHSD alone had no effect on neurons viability. Cell viability of neurons decreased significantly with sevoflurane treatment, compared with control group (P<0.01). Meanwhile, cell viability of sevoflurane-injured neurons increased significantly by RHSD in dose-dependent manner, compared with SEV group (P<0.05) (Figure 1A). The effect of 80 μM/ml was similar to that of 40 μM/ml RHSD (Fig.1A). Therefore, we excluded the 80 μM/ml RHSD group for the following assays. CFSE assay was conducted to verify the promotion function of RHSD with different concentrations (10, 20 and 40 μM/ml) on cell proliferative ability of hippocampal primary neurons treated with 3 % sevoflurane for 6 hr, showing that cell proliferative ability of neurons was inhibited significantly, with decreased M1 values, by sevoflurane in SEV group, compared with control group (P<0.01), which was promoted significantly, with increased M1 values, by RHSD in dose-dependent manners (10, 20 and 40 μM/ml) (P<0.05) (Figure 1B).

Rhodioloside inhibited cell apoptosis of sevoflurane-injured hippocampal neurons

Annexin V/PI double-stain and FCM assay were done to determine the effect of RHSD with different concentrations (10, 20 and 40 μM/ml) on cell apoptotic status of hippocampal neurons injured by 3 % sevoflurane for 6 hr. The results presented in Figure 2A suggested that sevoflurane remarkably facilitated cell apoptosis.
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of neurons in SEV group, compared with control
group (P<0.01), which was reduced significantly
by RHSD in dose-dependent manners (10, 20
and 40 μM/ml) (P<0.05). Expression levels of
apoptosis-related factors, such as Caspase-3,
Bax, Bcl-2, were determined by RT-qPCR in the
experimental groups. The results demonstrated
that sevoflurane dramatically up-regulated the
expression levels of pro-apoptotic factors,
Caspase-3 and Bax (P<0.01), and RHSD
effectively down-regulated them
dose-dependently (10, 20 and 40 μM/ml), both in
mRNA and protein manners (P<0.05).
Meanwhile, sevoflurane notably down-regulated
the expressions of apoptotic inhibitor, Bcl-2
(P<0.01), and RHSD effectively up-regulated
them, dose-dependently (10, 20 and 40 μM/ml),
both in mRNA and protein manners (P<0.05; Figure 2B).

**Rhodioloside activated cAMP/PKA signaling pathway in sevoflurane-injured hippocampal neurons**

The phosphorylation levels of cAMP/PKA in the
experimental groups were assessed by Western
blot. The results indicated that, protein levels of
p-PKA decreased significantly in SEV group,
compared with the control group (P<0.01), which
increased notably dose-dependently (10, 20
and 40 μM/ml) when treated with RHSD, compared
with SEV group (P<0.05). Meanwhile, there was
no significant difference in t-PKA levels among
different experimental groups (Figure 3A and B).

**Rhodioloside inhibited cell apoptosis of sevoflurane-injured hippocampal neurons.**

(A) Annexin V/PI double-stain and FCM assay were
performed to evaluate the effect of RHSD with
different concentrations (10, 20 and 40 μM/ml) on
cell apoptosis of neurons injured by 3 % sevoflurane for 6
hr. (B) Expression levels of apoptosis-related factors,
such as Caspase-3, Bax, Bcl-2, were determined by
RT-qPCR. ##: P<0.01 vs. Control group; *: P<0.05; **:
P<0.01 vs. SEV group; RHSD: Rhodioloside; FCM:
flow cytometry

**Figure 1:** Rhodioloside promoted cell viability of hippocampal neurons injured by sevoflurane. (A) CCK-8 assay was used to detect function of RHSD with different concentrations (10, 20, 40 and 80 µM/ml) on cell viability of 3 % sevoflurane treated neurons in different groups (Control, RHSD40, SEV, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV). (B) CFSE assay was conducted to verify the promotion function of RHSD on cell differentiation of neurons, after 6 hr treatment of sevoflurane. ###: P<0.01 vs. Control group; **: P<0.05; ***: P<0.01 vs. SEV group. RHSD: Rhodiolosides; SD: Sprague-Dawley; CCK-8: Cell Counting Kit-8; CFSE: carboxyfluorescein diacetate succinimidyl ester

**Figure 2:** Rhodioloside inhibited cell apoptosis of sevoflurane-injured hippocampal neurons. (A) Rhodioloside activated cAMP/PKA signaling pathway in hippocampal neurons injured by sevoflurane. The relative phosphorylation levels of PKA were assessed by Western blot in Control, SEV, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV groups. ##: P<0.01 vs. Control group; **: P<0.01; ***: P<0.01 vs. SEV group; RHSD: Rhodioloside

**Figure 3:** Rhodioloside activated cAMP/PKA signaling pathway in hippocampal neurons injured by sevoflurane. The relative phosphorylation levels of PKA were assessed by Western blot in Control, SEV, RHSD10+SEV, RHSD20+SEV, RHSD40+SEV groups. ##: P<0.01 vs. Control group; **: P<0.05; ***: P<0.01 vs. SEV group; RHSD: Rhodioloside
The function of rhodioloside was blocked by cAMP/PKA inhibitor in sevoflurane-injured hippocampal neurons

The FCM analysis on apoptotic rates verified that, RHSD decreased the apoptotic rates of hippocampal neurons treated with sevoflurane in RHSD40+SEV group, which was reversed by PKA inhibitor (P<0.05) (Figure 4A). RT-qPCR assays showed that the levels of active Caspase-3 and Bax were promoted and Bcl-2 was inhibited by PKA inhibitor in PKA inhibitor+RHSD40+SEV group, compared with RHSD40+SEV group (P<0.05) (Figure 4B).

DISCUSSION

Sevoflurane has been found to regularly cause behavioral and developmental cognitive maladies in children, which has necessitated research into finding remedies to avert its developmental anesthetic neurotoxicity [22,23]. Rhodiolosides are effectual to heighten neurogenesis in hippocampus and enhance cognition via anesthetic neurotoxicity [22,23]. Rhodiolosides are effectual to heighten neurogenesis in hippocampus and enhance cognition via apoptosis regulation in hippocampal neurons under stress. Verifying the likely molecular means of rhodioloside shielding neurons from sevoflurane injury is an exciting practice. The hippocampus is the fundamental tissue for spatial navigation and long-term memory in the brain. Sevoflurane (3 %) was used to develop an injury model in previous studies. Cell differentiation and the entire process of neuron development occurred together. In the current study, we found that rhodioloside promoted cell viability and prevented cell apoptosis of neurons damaged by 3 % sevoflurane dose-dependently (10, 20 and 40 μM/ml).

Sevoflurane neurotoxicity in undeveloped brain is linked to extreme apoptosis, including anomalous expression of Bcl-2 family members and caspases [18]. The members of Bcl-2 family, including Bax, Bcl-2 and Bcl-XL, are the primary apoptosis regulators [24, 25]. As an anti-apoptotic factor, Bcl-2 prevents apoptosis mostly by barring caspase pathway. On the contrary, Bax conjugates with Bcl-2, forms heterodimer to stimulate the discharge of cytochrome, activates caspase path and fosters apoptosis [26,27]. Caspases are a family of cysteine-aspartic proteases. Being the final executor of caspase pathway, caspase-3 plays an important role to cut down cellular components and induces apoptosis [28]. The findings of this study supported the results of other experiments by proving that sevoflurane-treatment on hippocampal neurons significantly increased the levels of expression of pro-apoptotic factors, such as Bax and Caspase-3, and reduced the levels of expression of anti-apoptotic factors such as Bcl-2. According to the findings, rhodioloside enhanced cell viability and differentiation, prevented apoptosis dose-dependently by weakening Bcl-2 and increasing Bax and Caspase-3 expression, in sevoflurane-injured hippocampal neurons, as expected.

Recent studies have proven that several pathways were involved in the injury of neurons by sevoflurane. The cAMP/PKA pathway is activated by a diverse stimulus, including growing factors, mechanical stress and so on. For signal transduction occurrence from the surface reflectors of cell membrane to the nucleus, PKA should be activated. According to research, cAMP/PKA signaling pathway plays a crucial role in the developmental neuronal endurance and sevoflurane neurotoxicity [6-8], to increase dendritic spine density during synaptogenesis in hippocampal neurons, and to help enhance learning and retention abilities. In this study, rhodioloside was discovered to promote cAMP/PKA activation effectively by increasing the levels of PKA phosphorylation, which was extraordinarily reduced by sevoflurane in hippocampal neurons.

PKA inhibitor lessened the function of rhodioloside on apoptosis inhibition in hippocampal neurons treated with sevoflurane. Consistent with the promoted apoptosis, the expression levels of Bax and active-Caspase increased, and Bcl-2 decreased when PKA inhibitor was present. Though, it would be better to examine the effect of PKA activation on it at the same time, the results have indicated that rhodioloside protected neurons from sevoflurane induced apoptosis through cAMP/PKA pathway, which might reduce cell viability and proliferative ability of primary hippocampal neurons.

CONCLUSION

The findings from this study suggest that rhodioloside promotes cell viability and proliferation, and prevents apoptosis of primary hippocampal neurons injured by sevoflurane, through cAMP/PKA pathway activation. Moreover, addition of PKA inhibitor deteriorated the function of rhodioloside by stimulating cell apoptosis. Our findings provided a novel evidence that rhodioloside could attenuate neurotoxicity of inhalational anesthetics in the clinics. The future direction to this study will be to investigate the effect of rhodioloside on sevoflurane-induced behavioral deviations, cognitive and memorial disorders in vivo.
DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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