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Original Research

The Role of the SIRT1-mTOR Signaling Pathway in Regulating Autophagy in Sevoflurane-Induced Apoptosis of Fetal Rat Brain Neurons

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Abstract

Background: Isoflurane is a commonly used general anesthetic widely employed in clinical surgeries. Recent studies have indicated that isoflurane might induce negative impacts on the nervous system, notably by triggering neuronal apoptosis. This process is pivotal to the development and emergence of neurological disorders; its misregulation could result in functional deficits and the initiation of diseases within nervous system. However, the potential molecular mechanism of isoflurane on the neuronal apoptosis remains fully unexplored. This study aims to investigate the regulatory role of the sirtuin 1-mechanistic target of rapamycin (SIRT1-mTOR) signaling pathway in autophagy during isoflurane-induced apoptosis of fetal rat brain neuronal cells. **Methods**: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, real-time quantitative polymerase chain reaction (qPCR), and Western blot were utilized to evaluate the apoptotic status of hippocampal tissue cells in fetal mice after sevoflurane exposure. Our further investigation was commenced with flow cytometry, immunofluorescence, qPCR, and Western blot to determine the impact of autophagy on sevoflurane-induced apoptosis in these neurons. On the other hand, we conducted an additional set of analyses, including flow cytometric analysis, qPCR, and Western blot, to further elucidate the neuroprotective potential of autophagy in neural cells of fetal mice subjected to sevoflurane-induced apoptosis. **Results**: Our findings indicated that a 3% sevoflurane treatment led to a significant rise in apoptosis among fetal rat hippocampal tissue cells and neurons. Levels of apoptosis-associated proteins, cleaved-caspase-3 and Bcl-2 associated X protein (Bax), were found to be markedly higher, coinciding with an enhancement in autophagy as evidenced by increased microtubule-associated proteins 1A/1B-light chain 3 (LC3) and decreased p62 expression. Concurrently, there was a notable up-regulation of sirtuin 1 (SIRT1) and a down-regulation of mechanistic target of rapamycin (mTOR) expression. In conclusion, our research elucidated the pivotal function of cellular autophagy in an apoptosis induced by sevoflurane in fetal rat nerve cells. Through experimental manipulation, we observed that interference with SIRT1 resulted in a reduction of both cleaved-caspase-3 and Bax levels. This intervention also beget a diminished expression of the autophagy-associated factor LC3 and an up-regulation of p62. Furthermore, inhibition against mTOR reversed the effects induced by SIRT1 interference, suggesting a complex interplay amid these regulatory pathways. **Conclusions**: SIRT1 possesses a capacity to modulate apoptosis in the hippocampal neurons of fetal rats triggered by sevoflurane, with mTOR functioning as an inhibitory factor within this signaling pathway.

Keywords: SIRT1-mTOR signaling pathway; sevoflurane; nerve cells autophagy; cerebral nerve cell apoptosis

1. Introduction

Sevoflurane is a widely applied clinical anesthetic known for its rapid onset, quick recovery, and stable therapeutic effects [1]. Nevertheless, studies have indicated that inhalation of anesthetics by neonates would ultimately result in acute neural inflammation and a significant increase in rapid apoptosis of neural cells, subsequently leading to cognitive impairments [2–4]. Extensive preclinical research has suggested that repeated exposure to sevoflurane in neonatal mice and monkeys brings about cognitive deficits and neurotoxicity [5–9]. Therefore, delving into the impact and mechanisms of sevoflurane anesthesia on brain neural cells has crucial clinical significance.

Autophagy and apoptosis are two common and crucial cellular biological processes. Under normal circumstances, autophagy serves to eliminate aberrant protein aggregates or damaged organelles within cells, providing protection to neuronal cells [10]. Literature report indicated that autophagy is of significance in neurodegenerative diseases namely Alzheimer's, Parkinson's, Huntington's, neuronal excitotoxicity, and brain ischemia [11]. Apoptosis is instigated via a spectrum of intrinsic and extrinsic mechanisms, encompassing mitochondrial-associated pathways and regulatory cell death pathways. In brain neurons, apoptosis significantly influences neural development, selective neuronal survival, and the occurrence of neurodegenerative

diseases. Aberrant regulation of apoptosis may contribute to the development of neurodegenerative diseases, such as Parkinson's disease, stroke, and brain injury. Autophagy and apoptosis exhibit interrelated and reciprocal biological functions in brain neurons. By clearing harmful components and damaged organelles within cells, autophagy promotes cell survival and functional stability, thereby inhibiting apoptosis. However, dysregulation of autophagy is implicated in exacerbating cellular death and apoptotic events. Concurrently, apoptosis is reciprocally influenced by autophagy, with discrete signaling molecules assuming pivotal roles in the orchestration of these interrelated cellular processes.

Sirtuin type 1 (SIRT1) is the mammalian homolog of yeast silent information regulator 2 (Sir2) and belongs to the third class of histone deacetylases. It has been shown to extend the lifespan of lower organisms, inhibit age-related myocardial diseases, and protect the mouse heart from oxidative stress [12]. Additionally, accumulating evidence demonstrates that SIRT1 participates in neuronal cell autophagy within the brain [13]. SIRT1 regulates autophagy through the interaction with the autophagy-related genes autophagy related 5 (Atg5), autophagy related 7 (Atg7), and autophagy related 8 (Atg8), thereby inducing their deacetylation [14]. Mechanistic target of rapamycin (mTOR) is a suppressor of cellular autophagy, and an inhibition against this molecule is known to enhance autophagic activity [15]. Interplay between SIRT1 and mTOR has been shown in prior research to orchestrate cellular autophagy, thereby contributing to neuroprotective mechanisms within the brain. Chen *et al*. [16] have discovered that Urolithin A promotes autophagy through activating the SIRT1 signaling pathway and inhibiting the mTOR signaling pathway, which makes for the prevention of D-galactose-induced brain aging by activating the miR-34a-mediated SIRT1/mTOR signaling pathway for neuroprotection. Wang *et al*. [17] discovered that nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) induces autophagy by regulating the SIRT1/mTOR pathway, thereby protecting aged rats from damage caused by acute ischemic stroke. However, precise impact of the SIRT1/mTOR pathway on cell autophagy and its influence on neuronal apoptosis following sevoflurane inhalation in fetal rats remains to be elucidated. This study demonstrated the mechanism of action of the SIRT1-mTOR signaling pathway in regulating cell autophagy in sevoflurane-induced apoptosis of hippocampal neurons in fetal rats.

2. Materials and Methods

2.1 Construction of Animal Models

Eight male and sixteen female specific pathogen free (SPF)-grade sprague dawley (SD) rats (aged 7–8 weeks) were provided by Chongqing Ensville Biotechnology Co., Ltd. (Chongqing, China). All the rats were maintained under standardized conditions with a temperature of 23 $\pm$ 2 °C, a humidity range of 35–60% and a 12-hour light-dark cycle. Those sixteen female rats were evenly assigned to two groups, comprising eight rats per group. Each group of female rats was co-housed with four male rats, and the vaginal plugs were removed as successful pregnancy. Anesthesia was administered on the 18th day of pregnancy. Rats of experimental group were placed in a self-made anesthesia box (50 cm $\times$ 50 cm $\times$ 30 cm) and connected to an anesthesia machine. 3% sevoflurane concentration was delivered with an oxygen flow rate of 2 L/min and an oxygen concentration of 50%, and inhalation was performed for four hours. The rats in the control group were only placed in the anesthesia box and given the same oxygen flow for four hours. All pregnant rats received continuous treatment for three days. After the third day of anesthesia, rats were euthanized by intraperitoneal injection of 3% sodium pentobarbital at a dose of 150 mg/kg, and then the fetal rats were taken for subsequent experiments.

Carefully remove the skull using straight and curved forceps, exposing the brain. Under a stereomicroscope, gently dissect the cerebral cortex using straight forceps to expose the hippocampal tissue. Carefully separate the hippocampal tissue from the surrounding brain tissue. Carefully remove the hippocampal tissue using curved forceps. Place the extracted hippocampal tissue in a container with phosphate buffered saline (PBS) buffer for washing and subsequent experiments.

All aninmal experiments were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Medical Research Ethics Committee of Children's Hospital of Chongqing Medical University (No. (142) in 2023).

2.2 Isolation and Culture of Fetal Rat Hippocampal Neuron Cells

Extraction of hippocampal neurons from fetal rats was followed by their cultivation, with all procedures executed as detailed below: Hippocampus obtained from the fetal rat were cut up fully with ophthalmic scissors and were added to the 0.125% of trypsin (S310KJ, Beyotime, Shanhai, China) for incubation for 30 min at 37 °C. The digestion was terminated by the addition of dulbecco's modified eagle medium (DMEM) (L110KJ, Basalmedia, Shanghai, China) containing 10% fetal bovine serum (FBS; AC03L055, Life-iLab, Shanghai, China). The cells were centrifuged, resuspended, and filtered until a uniform cell suspension was formed. Extracted cells were cultured in Neurobasa medium (21103049, Gibco, Grand Island, NY, USA) supplemented with 2% B27 and 10% FBS (AC03L055, Life-iLab) at 37 °C with 5% CO_2. On day 3 of inoculation, 5 µmol/L cytarabine was added to the culture plates to inhibit growth of non-neural cells. The medium was changed every 3 days and neuron cell growth was observed under an inverted microscope (BLD-200, BJCOSSIM, Beijing, China). Flow cytometry analysis was applied

to detect and identify fetal rat hippocampal neuron cells (**Supplementary Fig. 1**). Testing of the neuron cells for mycoplasma was negative.

2.3 Group Treatment of Fetal Rat Hippocampal Neurons with SIRT1 Inhibitor

Healthy fetal rat hippocampal neurons were harvested and digested using 0.25% trypsin solution to collect cells and prepare a cell suspension. Cell density was adjusted to 2×10^5 cells/mL for plating according to experimental requirements. After cells adhered, they were divided into groups as follows: (A) Control group: Cells were cultured in a 95% air and 5% carbon dioxide environment for 24 hours. (B) 3% sevoflurane group: Cells were cultured normally for 24 hours followed by exposure to 3% sevoflurane for 12 hours. (C) 3% sevoflurane + SIRT1 inhibitor group: Cells were cultured in complete medium containing 38 nM SIRT1 inhibitor (abs42027291, Absin, Shanghai, China) for 24 hours, followed by exposure to 3% sevoflurane for 12 hours. (D) 3% sevoflurane + SIRT1 inhibitor + rapamycin group: Cells were cultured in complete medium containing 38 nM SIRT1 inhibitor for 24 hours, then exposed to a medium containing 3% sevoflurane and 12.5 nM rapamycin for 12 hours. After completing the treatments, samples were collected for analysis.

2.4 TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining of fetal rat hippocampal tissue was performed upon application of 3,3'-Diaminobenzidine (Streptavidin-Horseradish Peroxidase) Tunel Cell Apoptosis Detection Kit (G1507, Servicebio, Wuhan, China) according to the instructions of the manufacturer. Briefly, those tissue slices were dewaxed and penetrated through Proteinase K working solution at 37 °C for 20 min. Each sample was added into Equilibration Buffer and incubated in Terminal deoxynucleotidyl transferase (TdT) incubation buffer at 37 °C for 1 h. Subsequently, pre-diluted streptavidin-Horseradish Peroxidase (HRP) reaction solution was added into each tissue. Following sample washing, the slides were dyed by Diaminobenzidine (DAB) chromogenic solution and were added into hematoxylin. The slides were sealed with a neutral resin. Finally, images were obtained through the inverted microscope (MF53, Mshot, Guangzhou, China).

2.5 Quantitative Polymerase Chain Reaction (qPCR) Analysis

Concentration and purity of RNA were measured in each sample via spectrophotometry after total RNA extraction. Subsequently, a reverse transcription kit (TSK302M, Tsingke, Beijing, China) was used for cDNA synthesis, and fluorescence quantitative PCR detection was performed through 2 ×T5 Fast qPCR Mix (SYBR Green I) (TSE002, Tsingke, Beijing, China) based on the instructions of the manufacturer. Primer sequences were presented in Table 1. Relative quantitative analysis was conducted with the $2^{-\Delta\Delta CT}$ method with *GAPDH* as an internal reference.

Table 1. Primer sequences.

Primers (organism: rat)	Sequences
cleaved-caspase-3-F	GAGCTTGGAACGCGAAGAAA
cleaved-caspase-3-R	TTGCGAGCTGACATTCCAGT
Bax-F	CGTCTGCGGGGAGTCACG
Bax-R	AGCCATCCTCTCTGCTCGAT
Sirt1-F	CTACCGAGACAACCTCCTGTT
Sirt1-R	GTCACTAGAGCTTGCGTGTG
Mtor-F	GCAATGGGCACGAGTTTGTT
Mtor-R	AGTGTGTTCACCAGGCCAAA
p62-F	GAGGCACCCCGTAAAGTCAT
p62-R	GATTCAACCGCCATGTGCTT
Lc3-F	CCAGGATTCAGCACCAGATGT
Lc3-R	CCTTGGCTACCTGGACTGTT
Gapdh-F	TGGTGCTGAGTATGTCGTGG
Gapdh-R	GGCGGAGATGATGACCCTTT

Sirt1, Sirtuin type 1; *Bax*, Bcl-2-associated X protein; *Mtor*, Mammalian target of rapamycin; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Lc3*, Microtubule-associated protein 1A/1B-light chain 3.

2.6 Western Blot (WB) Analysis

After extracting 500 µg of total proteins from the samples and mixing them with 5 × SDS loading buffer at 4:1 ratio resulting in a concentration of approximately 3.3 µg/µL, proteins were denatured by heating in a metal bath, followed by loading 60 µg of denatured total proteins onto the gel for electrophoresis. After electrophoretic separation of the bands, the proteins were transferred onto the Polyvinylidene fluoride (PVDF) membrane.Subsequently, the PVDF membranes were blocked in 5% skim milk and was incubated with primary antibodies anti-cleaved-caspase-3 (1:1000, A19654, ABclonal, Wuhan, China), anti-Bax (1:1000, A19684, ABclonal), anti-SIRT1 (1:1000, A19667, ABclonal), anti-mTOR (1:1000, A2445, ABclonal), anti-p62 (1:1000, A19700, ABclonal), anti-microtubule-associated protein light chain 3II (LC3II) (1:1000, R381544, ZENBIOSCIENCE, Chengdu, China) and GAPDH (1:1000, A19056, ABclonal) at 4 °C overnight. After washing with Tris Buffered Saline with Tween (TBST), the membranes were incubated with secondary antibody HRP Goat Anti-Rabbit Immunoglobulin G (IgG) (1:2000, AS014, ABclonal) at 37 °C for 1 h. An Enhanced Chemiluminescence (ECL) exposure solution (34580, Thermo, Waltham, MA, USA) was mixed at a 1:1 ratio of solution A to solution B, evenly spread over the entire membrane, and exposed for 1 min. The membranes were then placed in a nucleic acid and protein gel imaging system (Universal Hood II, Bio-Rad, Hercules, CA, USA) for exposure detection.

2.7 Immunofluorescence

After fixation, cell coverslips were washed with PBS three times, 5 min per round. A suitable amount of 0.3% Triton X-100 (ST795, Beyotime, Beijing, China) permeabilization solution was added to coverslips and incubated. These coverslips were then rinsed three times. Goat serum (C0265, Beyotime, Beijing, China) was added for blocking and then removed without rinsing. Primary antibody against SIRT1 (1:200, A0230, ABclonal, Wuhan, China) was used for incubation overnight at 4 °C. After washing three times with PBS, the specimens were cultured with secondary antibodies Cy3 Goat Anti-Rabbit IgG (H+L) (1:50, AS008, ABclonal, Wuhan, China) and FITC Goat Anti-Rabbit IgG (H+L) (1:50, AS011, ABclonal, Wuhan, China) in the dark, and followed by another three rounds of phosphate buffered saline (PBS) washing. 4',6-diamidino-2-phenylindole (DAPI) (C1005, Beyotime, Beijing, China) was provided and the cells were cultured again to stain nuclei. Excess DAPI was removed by washing with PBS three times. The coverslips were sealed with an anti-fluorescence quencher. Image acquisition was performed via an Mshot MF53 inverted microscope (MF53, Mshot, Guangzhou, China).

2.8 Flow Cytometry

Annexin V Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI)-apoptosis kit (40302ES50, YEASEN, Shanghai, China) was applied to detect the cell apoptosis by flow cytometry. Cells were seeded in the 6-well plate and incubated in the CO_2 incubator. When the cell density reached 70%, cells were added into FITC-dextran (1 mg/mL) and incubated for 2 h. Subsequently, cells were treated with the following procedures: PBS washing, trypsin digestion, and centrifugation after neutralizing with serum-containing medium. The supernatant was abandoned and cell pellets were resuspended in PBS. Finally, flow cytometry was performed using a CytoFLEX flow cytometer (CytoFLEX, Beckman, Brea, CA, USA).

2.9 Statistical Analysis

Data analyses and plotting were performed using the GraphPad Prism 8.01 software (GraphPad Software Inc., San Diego, CA, USA). The data are presented as mean $\pm$ standard error. The *t*-test was used for the comparison between the two groups, while ANOVA was applied for comparisons among multiple groups. Post hoc tests were performed using Tukey's method. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Inhalation of Sevoflurane Induces Apoptosis of Hippocampal Tissue in Fetal Rats

Outcomes of TUNEL assay (Fig. 1A) showcased a notable increase in the rate of cell apoptosis within the hippocampal tissue of fetal rats exposed to 3% sevoflurane, when compared to the control. Results from qPCR and western blot (Fig. 1B,C) revealed that in 3% sevoflurane treatment group, the mRNA and protein expression of cleaved-caspase-3, Bax, and SIRT1 were substantially elevated ($p < 0.05$, $p < 0.001$), while the mRNA and protein expressions of mTOR were greatly reduced ($p < 0.01$, $p < 0.001$). All thoses findings implied that sevoflurane treatment facilitated an up-regulation of both apoptosis-related proteins, cleaved-caspase-3 and Bax, along with an increase in SIRT1 expression; concurrently, there was a down-regulation of mTOR expression. Immunofluorescence analysis further confirmed the up-regulation of SIRT1 expression (Fig. 1D). SIRT1 protein level was significantly increased in 3% sevoflurane treatment group, which was consistent with our qPCR and western blot results. These findings implied that sevoflurane treatment might participate in cell apoptosis in fetal rat hippocampal tissue by regulating the expression of these factors, potentially serving as a crucial factor contributing to cognitive dysfunction.

3.2 Promoting Effect of Sevoflurane on Apoptosis and Autophagy of Fetal Rat Hippocampal Neurons

In 3% sevoflurane treatment group, apoptotic levels of fetal rat hippocampal neurons were markedly higher than the control, as indicated by the flow cytometry results (Fig. 2A). This suggested that sevoflurane treatment could lead to an increase in neuronal apoptosis, potentially exerting adverse effects on cognitive functions. Immunofluorescence detection results (Fig. 2B) revealed an apparent elevation in SIRT1 protein expression in 3% sevoflurane treatment group. SIRT1 functions as an essential regulator of cellular autophagy, and its increased expression during sevoflurane therapy likely signifies the activation of autophagic processes. Further qPCR and WB analysis results (Fig. 2C,D) demonstrated a substantial increase ($p < 0.01$, $p < 0.001$) in the mRNA and protein expression of cleaved-caspase-3, Bax, and SIRT1 in hippocampal neurons of fetal rats exposed to sevoflurane. Conversely, mRNA and protein expressions of mTOR were found greatly reduced ($p < 0.05$, $p < 0.001$). Simultaneously, alterations in the expression of autophagy-related genes and proteins were observed, specifically the up-regulation of LC3 expression and down-regulation of p62 expression.

3.3 Neuroprotective Effect of Autophagy on Fetal Rat Nerve Cell Apoptosis Induced by Sevoflurane Inhalation Anesthesia

In this section, we employed strategies of SIRT1 disruption and mTOR suppression to evaluate effects of autophagy on sevoflurane-induced apoptosis in fetal rat hippocampal neurons. Flow cytometric analysis revealed (Fig. 3A) that hippocampal neuron cell apoptosis decreased in 3% sevoflurane + SIRT1 inhibitor group compared to 3% sevoflurane group, suggesting that SIRT1 interference is likely to produce a protective effect against sevoflurane-

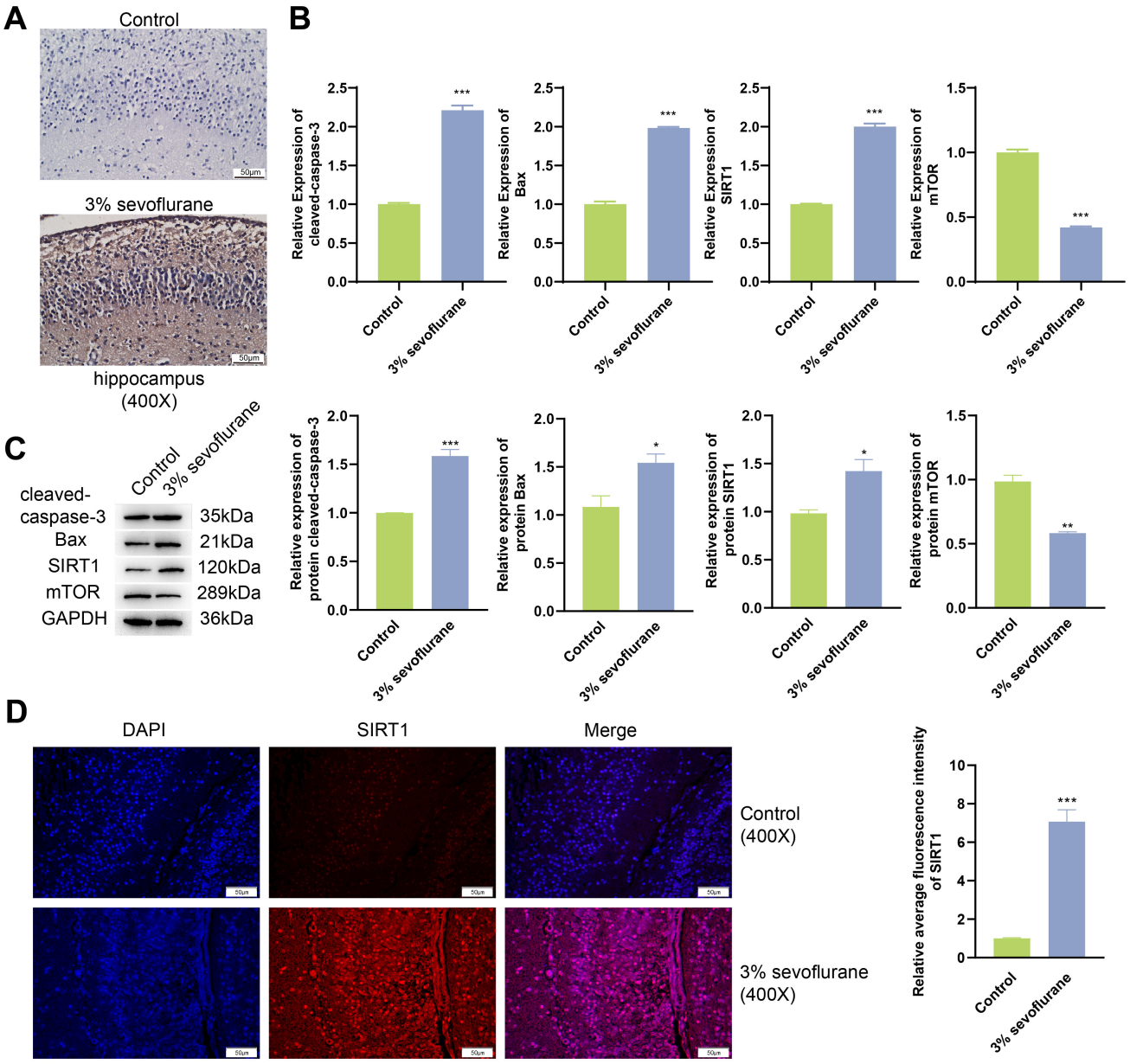

Fig. 1. Inhalation of sevoflurane begets apoptosis of hippocampal tissue in fetal rats. (A) Fetal rat hippocampal tissuel apoptosis was assessed through Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis. Scale bars, 50 µm. (B) Real-time quantitative polymerase chain reaction (qPCR) analysis was conducted on fetal rat hippocampal tissue for cleaved-caspase-3, Bax, Sirtuin 1 (SIRT1), and mechanistic target of rapamycin (mTOR). (C) Western blot analyses of fetal rat hippocampal tissue for cleaved-caspase-3, Bax, SIRT1, and mTOR. (D) Immunofluorescence combined with 4',6-diamidino-2-phenylindole (DAPI) staining was utilized to examine expression of SIRT1 in fetal rat hippocampal tissue. Scale bars, 50 µm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* control. n = 3.

induced neuronal apoptosis. In our mTOR inhibition experiment, rapamycin was employed to suppress mTOR activity; this approach resulted in a higher incidence of hippocampal neuron apoptosis in 3% sevoflurane + SIRT1 inhibitor + rapamycin group compared to 3% sevoflurane + SIRT1 inhibitor group (Fig. 3A). This observed increase implies that mTOR suppression potentially negates the neuroprotective impact of SIRT1 knockdown, thereby intensifying sevoflurane-induced neuronal apoptosis. Immunofluorescence detection (Fig. 3B) implied an apparent decrease in the expression of SIRT1 in both 3% sevoflurane + SIRT1 inhibitor and 3% sevoflurane + SIRT1 inhibitor + rapamycin groups. Moreover, qPCR and WB results (Fig. 4A,B) further supported these findings. In 3% sevoflurane + SIRT1 inhibitor group, expression of SIRT1 significantly decreased, along with a significant decrease in cleaved-caspase-3, Bax, and LC3II expression ($p < 0.01$, $p < 0.001$), while that of mTOR and p62 sub-

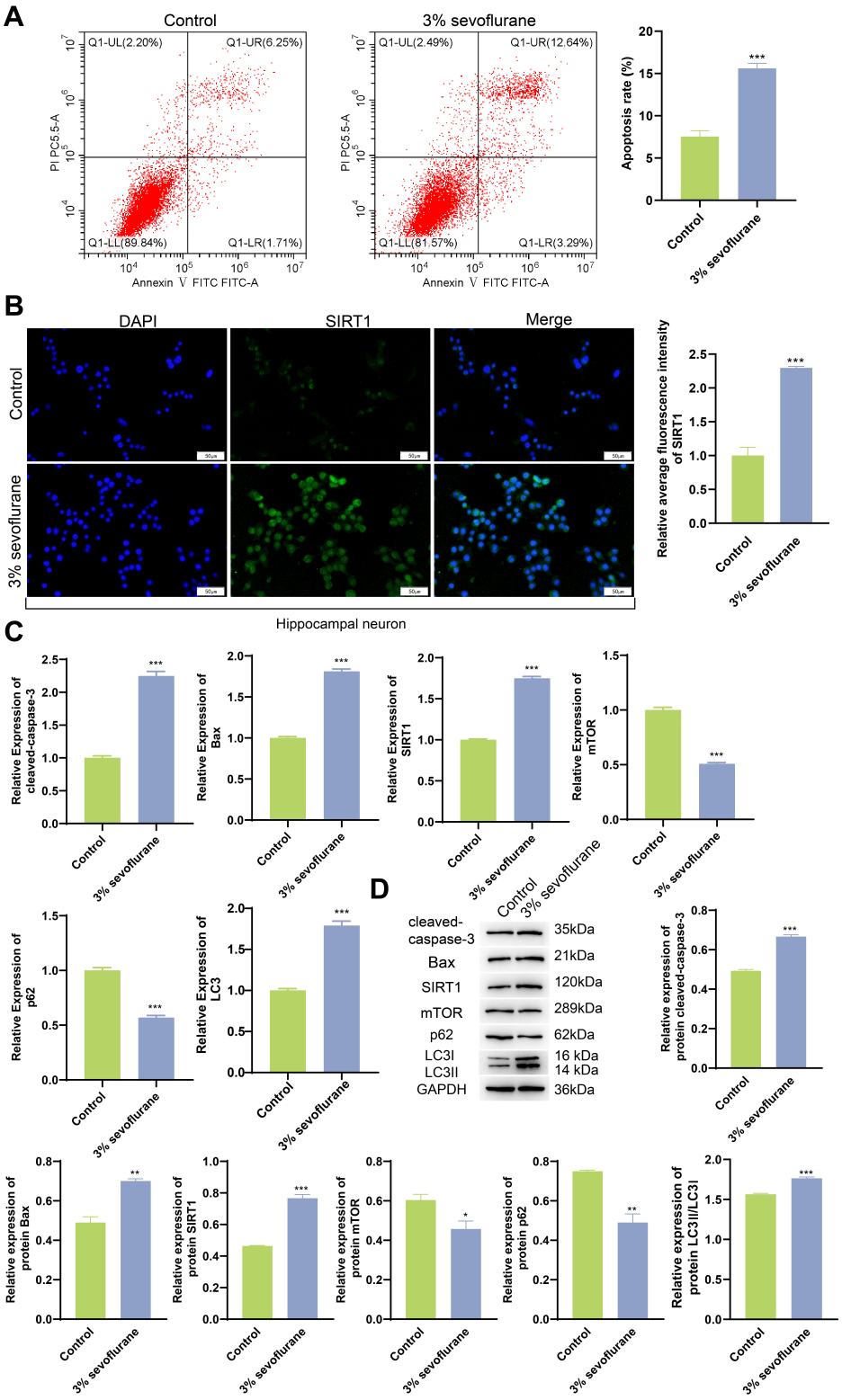

Fig. 2. Promoting effect of sevoflurane on apoptosis and autophagy of fetal rat hippocampal neurons. (A) Changes in apoptosis of fetal rat hippocampal neuron cells after 3% sevoflurane treatment. (B) The expression of SIRT1 was detected by immunofluorescence. Scale bars, 50 μm. (C) qPCR analysis of *cleaved-caspase-3*, *Bax*, *SIRT1*, *mTOR*, *p62*, and *LC3II* in fetal rat hippocampal neurons. (D) Western blot analyses of cleaved-caspase-3, Bax, SIRT1, mTOR, p62, and microtubule-associated protein light chain 3II (LC3II) in fetal rat hippocampal neurons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs.* control. n = 3. UL: Upper Left; UR: Upper Right; LL: Lower Left; LR: Lower Right.

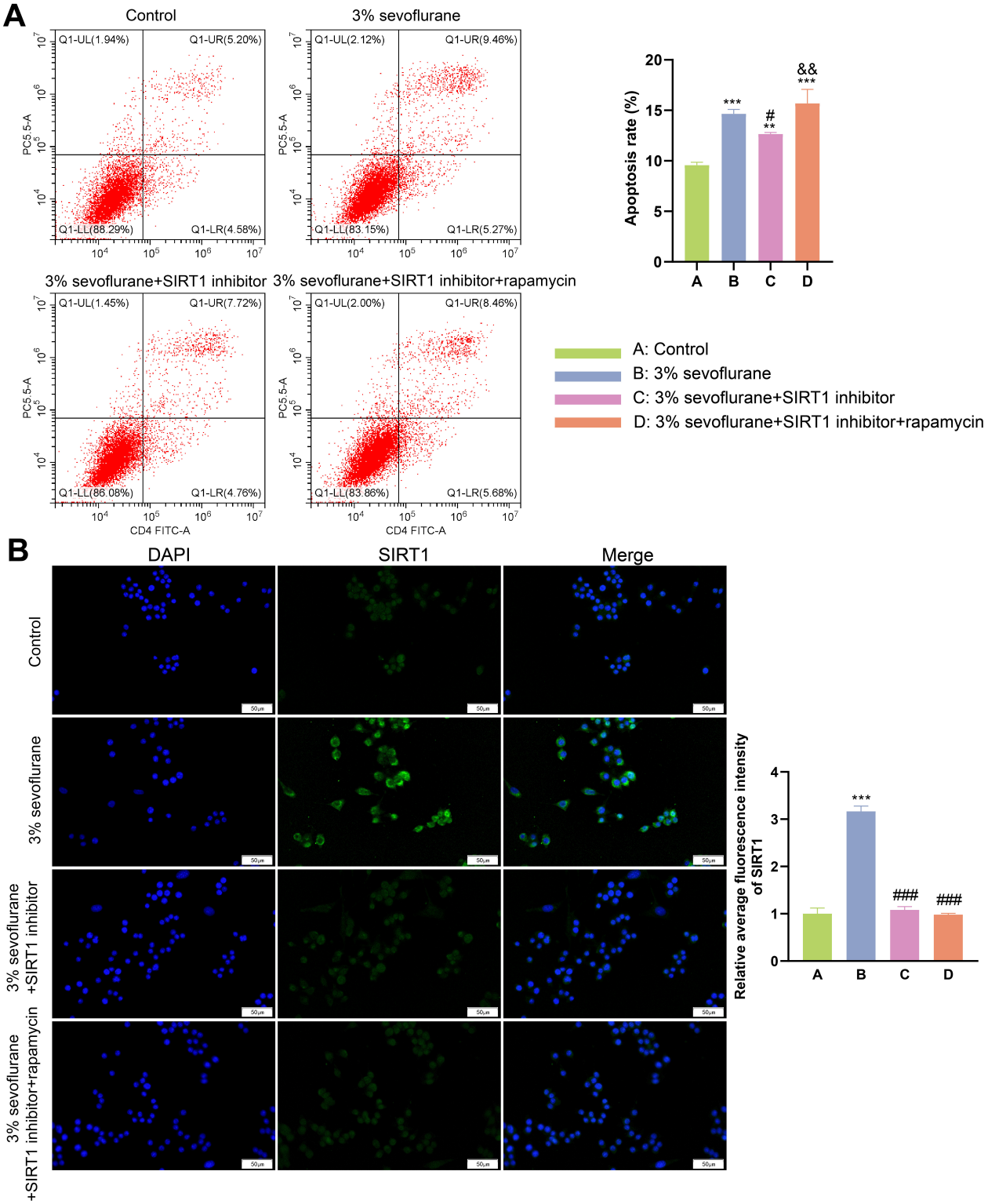

Fig. 3. Autophagy regulates apoptosis of hippocampal neurons in fetal rats induced by sevoflurane inhalation anesthesia. (A) Changes in apoptosis of fetal rat hippocampal neuron cells after different treatment. (B) The expression of SIRT1 was detected by immunofluorescence after different treatment. Scale bars, 50 µm. Statistical significance compared to the control group: $^{**}p < 0.01$, $^{***}p < 0.001$; compared to 3% sevoflurane group: $\#p < 0.05$, $\#\#\#p < 0.001$; compared to 3% sevoflurane + SIRT1 inhibitor group: $\&\&p < 0.01$. n = 3.

stantially increased ($p < 0.001$). In 3% sevoflurane + SIRT1 inhibitor + rapamycin group, expression of cleaved-caspase-3, Bax, and LC3II significantly increased ($p < 0.01$, $p < 0.001$), while mTOR and p62 expression was

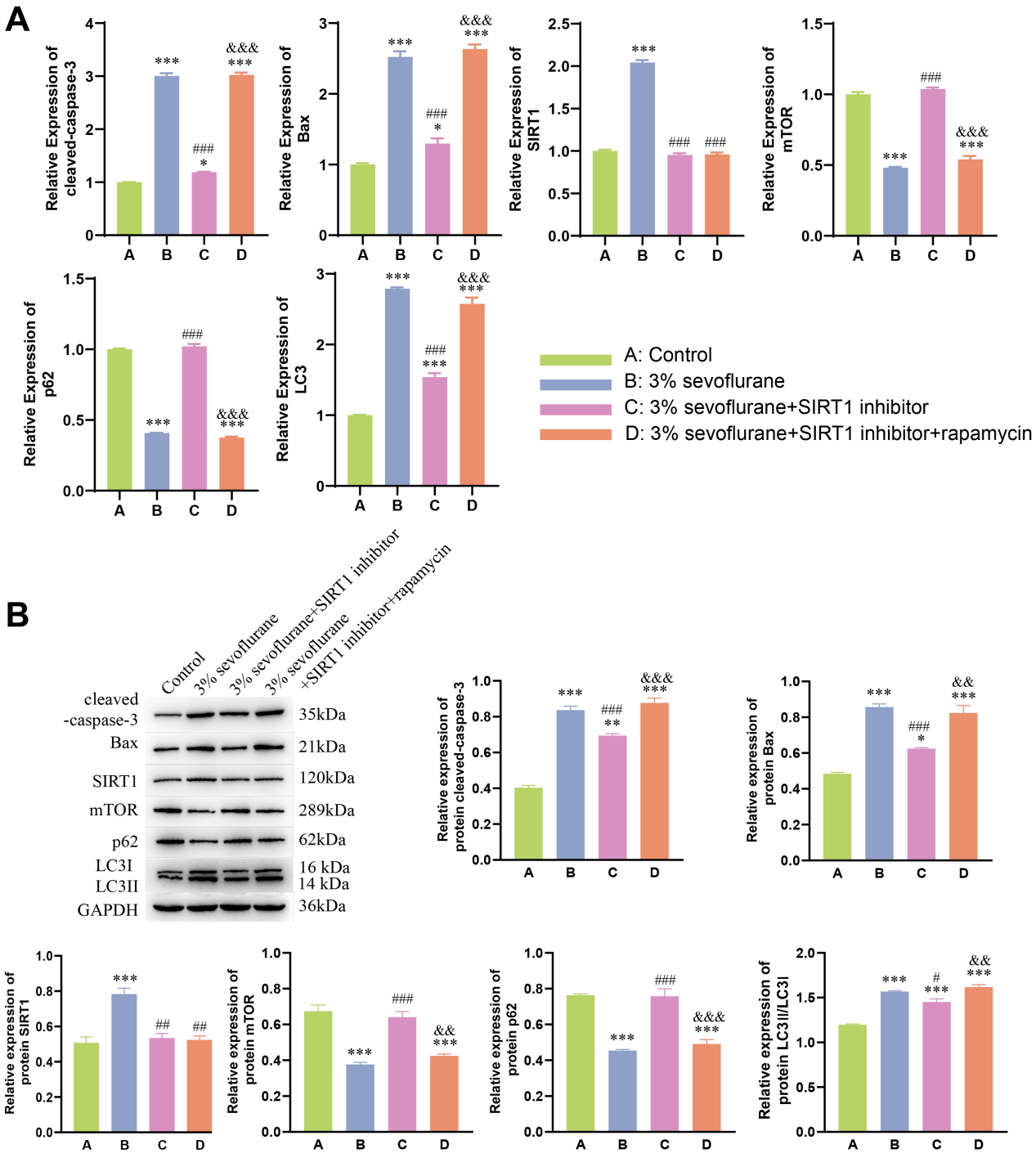

Fig. 4. Neuroprotective effect of autophagy on fetal rat nerve cell apoptosis induced by sevoflurane inhalation anesthesia. (A) qPCR analysis of *cleaved-caspase-3*, *Bax*, *SIRT1*, *mTOR*, *p62*, and *LC3II* in fetal rat hippocampal neurons. (B) Western blot analysis of cleaved-caspase-3, Bax, SIRT1, mTOR, p62, and LC3II in fetal rat hippocampal neurons. Statistical significance compared to the control group: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; compared to 3% sevoflurane group: $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$; compared to 3% sevoflurane + SIRT1 inhibitor group: $\&\&p < 0.01$, $\&\&\&p < 0.001$. n = 3.

significantly decreased ($p < 0.01$, $p < 0.001$). Our experimental outcomes have furnished compelling evidence that substantiates SIRT1's integral function in modulating sevoflurane-induced apoptosis in hippocampal neurons, concurrently highlighting mTOR's antagonistic regulatory influence within this biological cascade.

4. Discussion

Sevoflurane, a commonly used anesthetic in clinical settings, has detrimental effects on animal nervous system when inhaled at high concentrations for extended periods. Reports have suggested that prenatal exposure to sevoflurane inhibits the proliferation of fetal neural stem cells [18],

neuronal migration, and axonal growth [19]. This inhibition leads to neuronal apoptosis [20] and a sustained imbalance between excitatory and inhibitory neurons in the medial prefrontal cortex [21], resulting in learning and memory deficits as well as anxiety-like behavior. Our study findings indicated an increase in apoptosis of fetal rat hippocampal neurons following sevoflurane treatment, potentially serving as a significant factor contributing to cognitive dysfunction. Apoptosis, a normal physiological process in the body, serves as a self-regulated programmed death mechanism, allowing to eliminate damaged or unnecessary cells to maintain normal structure and function of the tissues and organs. However, an abnormal activation of apoptosis leads to various diseases, including neurological disorders. In this study, we initially observed that inhalation of sevoflurane during fetal development resulted in increased apoptosis of hippocampal neurons in fetal rats. TUNEL experiment results showed a significantly higher apoptosis level in sevoflurane-treated group compared with the control group, supporting detrimental impact of sevoflurane on neural cells in fetal rats. Additionally, qPCR and western blot revealed a significant up-regulation in the expression of cleaved-caspase-3 and Bax in the 3% sevoflurane-treated group. Xu *et al*. [22] demonstrated increased levels of cleaved-Caspase-3 and Bax in HCN-2 cells after sevoflurane treatment. Similarly, Areias *et al*. [23] found a notable increase in cleaved-caspase-3 after sevoflurane treatment at 1.63 [1.56–1.77]% of brain volume. The results suggested that the developing brain is highly susceptible to anesthesia exposure, leading to an increase in cell apoptosis, as evidenced by a significant increase in SIRT1 expression and a marked decrease in mTOR expression.

Additionally, this study has elucidated the involvement of autophagy in neuronal apoptosis triggered by sevoflurane treatment. Autophagy functions as an essential intracellular process for the degradation and recycling of cellular components, crucial for maintaining metabolic equilibrium within the cell. Our experimental results indicated an up-regulation of LC3 expression and a simultaneous down-regulation of p62 expression in sevoflurane-treated group. Wang *et al*. [24] found increased levels of LC3II/LC3I and decreased p62 in hippocampal neurons treated with sevoflurane. Zhou *et al*. [25] suggested an increase in the expression of autophagy marker protein LC3 and a decrease in the protein p62 in H4 human glioma cells after 6 h of exposure to 4.1% sevoflurane. These findings confirmed that sevoflurane exposure induces endoplasmic reticulum stress and activates autophagy in H4 human glioma cells, providing negative feedback to inhibit sevoflurane-induced cell apoptosis. Additionally, SIRT1 functions as a pivotal modulator of autophagy [26], with its augmentation being possibly linked to heightened autophagic activity. Conversely, mTOR operates as an inhibitory factor in autophagy regulation, and its suppression is likely to further stimulate an upsurge in autophagic processes. Autophagy in sevoflurane-induced neuronal apoptosis was verified through SIRT1 knockdown and mTOR inhibition. Our results indicated a decreasing trend in the apoptosis of fetal rat hippocampal neurons in the SIRT1 interference experiment. Contrary to expectations, the mTOR inhibition experiment revealed an upward trajectory in rate of hippocampal neuronal apoptosis, indicating that mTOR suppression might potentially counteract the neuroprotective influence conferred by SIRT1 modulation and exacerbate sevoflurane-triggered neuronal cell death. This observation intimated that SIRT1 governs autophagic processes, thereby imposing a mitigating impact on neuronal apoptosis incited by sevoflurane, in opposition to mTOR's antagonistic regulatory stance. Therefore, autophagy and the SIRT1-mTOR pathway are potential targets in treating anesthesia-induced neuronal apoptosis and cognitive impairment.

In conclusion, our research delineated that exposure to sevoflurane via inhalation in fetal rats precipitates an escalation in neuronal apoptosis, with the modulatory factors SIRT1 and mTOR assuming critical factors in this cascade. Over-expression of SIRT1 is implicated in the sevoflurane-induced augmentation of cellular autophagy, thereby conferring a protective mechanism against neuronal apoptosis. In contrast, mTOR imparts an antagonistic regulatory influence within this scenario. Our research elucidated the ramifications of anesthetic agents on neuronal cells, thereby providing a foundation for the formulation of future investigative endeavors focused on neuroprotective strategies. However, more investigations remain necessary to figure out detailed regulatory mechanisms of cellular autophagy in sevoflurane-induced neuronal apoptosis, and to validate the clinical significance and application potential of these findings. Firstly, further exploring the precise molecular interactions within the SIRT1-mTOR pathway and its role in neuronal apoptosis induced by sevoflurane exposure is essential. Subsequently, future research should focus on the role of key autophagy-related genes such as Beclin1 and LC3 to investigate the relationship between autophagy and sevoflurane-induced apotopsis in fetal rat hippocampal neurons. Additionally, large-scale clinical studies are required to assess the relevance of these findings in human patients undergoing sevoflurane anesthesia. These comprehensive investigations will not only expand our understanding of sevoflurane neurotoxicity but also pave the way for the development of novel therapeutic strategies for preventing or treating sevoflurane-induced apoptosis in neural cells.

5. Conclusions

Sevoflurane treatment incites apoptosis in hippocampal neurons of fetal rats, wherein cellular autophagy wields a pivotal influence on the proceedings. Empirical research corroborates the notion that SIRT1 orchestrates apoptotic response of these neurons to sevoflurane exposure, concurrently with mTOR fulfilling an inhibitory regulatory function within this framework. These results hold critical significance for a thorough understanding of the mechanism

of action of sevoflurane on neural cells and the future development of relevant neuroprotective strategies.

Abbreviations

SIRT1, Sirtuin type 1; Bax, Bcl-2-associated X protein; mTOR, Mammalian target of rapamycin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LC3, Microtubule-associated protein 1A/1B-light chain 3.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

YHL conceived, designed, and performed the experiments, and wrote the original draft. CX and YT analyzed and interpreted the data. SJ designed the research, formal analysis, writing review and editing, and supervision. All authors contributed to the editorial changes in the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal experiments proposed in this study have been approved by the Medical Research Ethics Committee of Children's Hospital of Chongqing Medical University (No. (142) in 2023). The protocol for animal research outlined in this study complies with ethical guidelines and regulations regarding the humane and responsible use of animals in scientific research.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2909324.

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