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ORIGINAL RESEARCH

Alisol A attenuates Sevoflurane-induced hippocampal neuron injury *via* targeting SIRT3

Jing Hu[1], Tianyin Liu[2,*], Min Zeng[1]

[1]Department of Anesthesiology, Xinyu Hospital of Traditional Chinese Medicine, 338025 Xinyu, Jiangxi, China
[2]Department of Anesthesiology, The First Affiliated Hospital of Nanchang University, 330006 Nanchang, Jiangxi, China

***Correspondence**
Liutianyin_666@163.com
(Tianyin Liu)

Abstract

To investigate the effects of Alisol A on Sevoflurane (Sev)-induced neuroinflammation in hippocampal HT22 cells and explores the underlying mechanisms involving Sirtuin 3 (SIRT3) activation. Neuroinflammation was induced through the exposure of HT22 cells were exposed to 4% for 6 h. We evaluated cell viability through cell counting kit-8 (CCK-8) assays, apoptosis and ferroptosis through flow cytometry (FCM) and immunoblot assays. Alisol A effects at different concentrations (0, 2.5, 5, 10 and 20 μM) on Sev-induced cell damage were examined. Additionally, using the SIRT3 inhibitor 3-(Benzylamino)-3-Methylbutanoic Acid (3-TYP), SIRT3's role in mediating these effects was investigated. Alisol A significantly increased Sev-induced HT22 cell viability and inhibited Sev-induced apoptosis and ferroptosis. Alisol A treatment decreased levels of apoptotic markers (Bcl-2 associated X protein (Bax), cleaved caspase-3, cleaved poly ADP-ribose polymerase (PARP)) and ferroptosis markers (MDA, GSH), while increasing expressions of SIRT3 and ferroptosis inhibitors (glutathione peroxidase 4 (GPX4), Solute Carrier Family 7 Member 11 (SLC7A11)). Alisol A's protective effects were reversed by 3-TYP, confirming SIRT3 activation involvement. Alisol A mitigates Sev-induced neuroinflammation and cell damage in HT22 cells by activating SIRT3. Alisol A may therefore be a promising drug for treating Sev-induced neurotoxicity.

Keywords

Sevoflurane (Sev); Alisol A; Neuroinflammation; Ferroptosis; SIRT3

1. Introduction

Sevoflurane (Sev) is a widely used inhalation anesthetic with characteristics of low blood gas ratio, zero irritant odor, rapid onset, rapid dissipation and limited cardiopulmonary inhibition [1]. Sev has also become common in pediatric general anesthesia. However, Sev blocks neurogenesis, induces neuronal apoptosis and causes neuroinflammation [2]. Additionally, it inhibits neural progenitor cell proliferation, weakens neural stem cell self-renewal, and induces neuroinflammation in mouse microglia [3]. It is clear that Sev is adversely affecting the central nervous system. Detailed research on Sev's mechanism and further screening of effective treatment drugs is needed to further improve Sev-induced neuroinflammation.

Sirtuin3 (SIRT3) is a Class III histone deacetylases (HDAC) localized primarily in mitochondria, expressed in numerous tissues, and highly expressed in brain [4]. SIRT3, with its powerful deacetylase activity, plays a vital role in protecting organs during pathological states such as inflammation and oxidative stress. It contributes to metabolic diseases such as diabetes, neurodegenerative diseases, and other diseases [5]. SIRT3 activation improves cognitive decline induced by surgery/anesthesia in mice through antioxidant stress and hippocampal anti-inflammatory effects [6]. Sevoflurane (Sev) treatment increases ferritin levels in hippocampal neurons, but decreases GPX4 [7].

Alisma orientalis is widely used. Among its many pharmacological properties, Alisma purpurea extract possesses anti-inflammatory, liver protecting, anti-hepatitis B virus, antibacterial, anticancer properties [8]. One of the triterpenoids isolated from Alisol is Alisol A. It activates the Serine/threonine protein kinase B (AKT)/glycogen synthase kinase-3β (GSK3β) pathway, which plays a neuroprotective role in mice suffering from cerebral ischemia [9]. Alisol A protects against high fat diet-induces pathological brain aging [10]. However, the effect and mechanism of Alisol A on Sev-induced injury to hippocampal neuron remain unclear.

Here, we investigate how Alisol A may affect Sev-induced neuroinflammation. This study demonstrated that Alisol A attenuates Sev-induced cell damage in neurons *via* SIRT3 activation.

2. Materials and methods

2.1 Cell culture and treatment

Mouse hippocampus HT22 cell line (Procell Life Science & Technology Co., Ltd.) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine

serum (FBS) at a density of 1×10^6 cells per well in 6-well plates. For Alisol A treatment, HT22 cells were exposed to Alisol A (HY-N0853, MCE, Newark, NJ, USA) at concentrations of 0, 2.5, 5, 10 and 20 μM for 24 h. Alisol A was diluted in Dimethyl sulfoxide (DMSO). DMSO's final concentration in the culture medium did not exceed 0.1%. For Sev exposure, HT22 cells were treated with 4% Sev (purchased from Sigma, 28523-86-6, St. Louis, MO, USA, purity >98%) for 6 h to induce the neuroinflammation model. HT22 cells were detached from the culturing vessel with 0.25% trypsin-Ethylenediamine tetraacetic acid (EDTA) (25200-072, Invitrogen, Carlsbad, CA, USA) solution for passage and reseeding. HT22 cells were treated with SIRT3 inhibitor 3-TYP (HY-108331, MCE, Newark, NJ, USA) at 50 μM for 24 h. To account for any solvent effects, a vehicle control using the same DMSO concentration was included in the 3-TYP treatment.

2.2 Cell viability

HT22 cells were seeded into 96-well plates at a density of 1×10^4 cells per well in a final volume of 100 μL of culture medium per well. After Alisol A and/or Sev treatment as described, cell viability was assessed using CCK-8 assay (C0078, Beyotime, Shanghai, China). Each well was treated with 10 μL of CCK-8 reagent per 100 μL of medium. An additional 4 h cell incubation at 37 °C before measuring absorbance value at 450 nm.

2.3 Cell apoptosis

HT22 cells were digested into single cells and resuspended in a reaction buffer containing Annexin V and Propyl iodide (PI) (C1062, Beyotime, Beijing, China) for 5 min and avoid light. Cell apoptosis was analyzed by flow cytometer (BD FACSymphony™ A3, BD Biosciences, Franklin Lake, NJ, USA).

2.4 2′,7′-Dichlorofluorescein (DCF) staining

Following the indicated treatment, HT22 cells were fixed and blocked with goat serum for 1 h. Further incubation of slices with DCF detection kit (ab238535, Abcam, Cambridge, UK) was performed according to manufacturer's instructions. Photos were captured after washing in phosphate buffered saline (PBS).

2.5 Fe^{2+}, Malondialdehyde (MDA) and GSH detection

We isolated cells for Fe^{2+} (ab83366, abcam, Cambridge, UK), MDA (S0131S, Beyotime, Beijing, China) and GSH (A006-2-1, Jiancheng Bioengineering Institute of Nanjing, Nanjing, Jiangsu, China) detection using relevant commercial kits. Cells were homogenized and centrifuged for 20 min. The sample was gently shaken, mixed and covered for reaction for 2 h at 37 °C.

2.6 Quantitative Polymerase chain reaction assay

RNA was reverse-transcribed by a Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) reverse transcriptase (M1701, Promega, Madison, WI, USA). Quantitative PCR was conducted using a SYBR Ex Taq kit (638319, Takara, Kyoto, Japan). SIRT3 (F) 5′-GAGCGGCCTCTACAGCAAC-3′, (R) 5′-GGAAGTAGTGAGTGACATTGGG-3′. β-actin (F) 5′-GTCAGGTCATCACTATCGGCAAT-3′, (R) 5′-AGAGGTCTTTACGGATGTCAACGT-3′.

2.7 Immunoblot assay

Proteins were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and electrotransferred to poiy vinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% Bovine serum albumin (BSA). Subsequently, membranes were incubated with primary antibodies of Bax (1:1000, ab32503, Abcam), cleaved caspase-3 (1:1000, ab32042, Abcam), cleaved PARP (1:1000, ab32064, Abcam), GPX4 (1:1000, ab125066, Abcam), SLC7A11 (1:500, ab307601, Abcam), SIRT3 (1:500, ab217319, Abcam) and beta-actin (1:3000, ab8226, Abcam). Membranes were then cultured with secondary antibodies for 2 h. Enhanced chemiluminescence (ECL) kit was used to visualize membranes.

2.8 Statistics

Data analysis was performed using GraphPad 5.0 software (Graphpad plc., San Diego, CA, USA). Data were presented as mean ± Standard deviation (SD). The analysis among groups was conducted using One-way Analysis of Variance (ANOVA) and Tukey's HSD (Honestly Significant Difference). $p < 0.05$ indicates statistically significant differences.

3. Results

3.1 Alisol A increases Sev-induced HT22 cells' viability

To uncover Alisol A's possible effects on neurons, cell proliferation in response to increasing doses of Alisol A (0, 2.5, 5, 10 and 20 μM) in HT22 cells for 24 h was detected. Alisol A's molecular formula was shown in Fig. 1A. CCK-8 assays indicated that a high concentration of Alisol A (20 μM) decreases HT22 cell growth, resulting in a decreased Optical density (OD)450 value (Fig. 1B). Low concentration, however, had modest effects on HT22 cell viability (Fig. 1B). Therefore, in next assays, Alisol A at a low concentration was used. Alisol A effects on Sev-induced HT22 cells was detected. CCK-8 assays confirmed that Sev treatment decreases HT22 cell viability (Fig. 1C). However, Alisol A treatment (2.5, 5 and 10 μM) significantly reversed Sev-induced suppression of HT22 cell viability (Fig. 1C). Therefore, Alisol A increases Sev-induced neuronal survival.

3.2 Alisol A inhibits Sev-induced cell apoptosis

Since Alisol A increased the viability of neuron cell lines, its possible effects on the apoptosis of Sev-induced HT22 cells was detected. Through FCM assays, Sev treatment increased apoptosis neurons (Fig. 2A). Alisol A treatment, however, significantly reversed Sev-induced HT22 cell apoptosis (Fig. 2A,B). According to Immunoblot analysis, HT22 cells treated with Sev displayed increased expression of Bax and cleaved caspase 3, while Alisol A reduced these expressions, suggesting suppression of apoptosis (Fig. 2C). Alisol A also reversed Sev-induced cleaved PARP expression in HT22 cells (Fig. 2D). Therefore, Alisol A inhibits Sev-induced cell apoptosis.

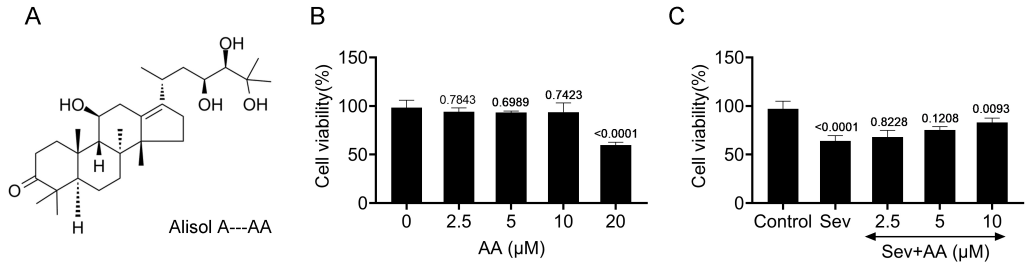

FIGURE 1. Alisol A increases Sev-induced HT22 cell viability. (A) The molecular formula for Alisol A. (B) Alisol A's effects on HT22 cell growth were evaluated by CCK-8 assays after AA treatment at 0, 2.5, 5, 10 and 20 μM for 24 h. OD450 value was measured. (C) Alisol A's effects on HT22 cell growth after Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. OD450 value was measured. Sev: Sevoflurane; AA: Alisol A.

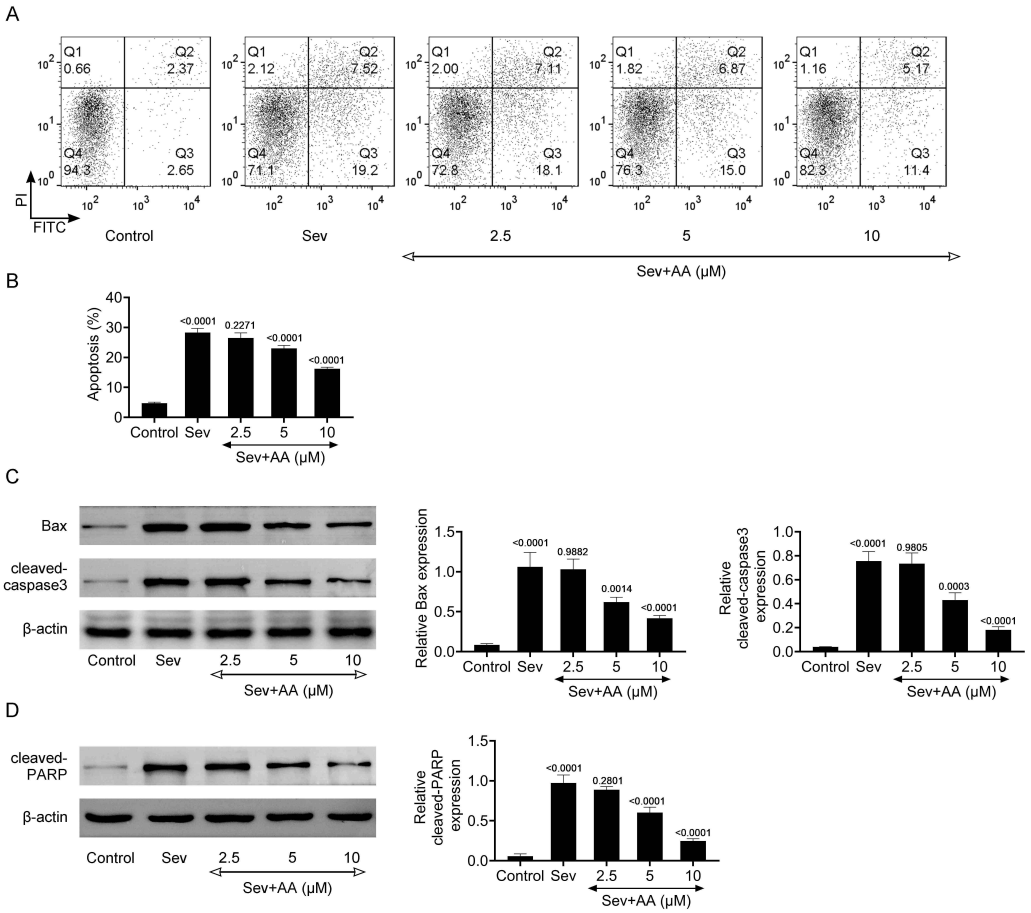

FIGURE 2. Alisol A inhibits Sev-induced cell apoptosis. (A) FCM assays showed HT22 cell apoptosis degree upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. (B) Panel A quantification. (C) Immunoblot assays demonstrated Bax expression and cleaved caspase 3 in HT22 cells upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. Protein expressions were quantified. (D) Immunoblot assays showed the expression of cleaved PARP in HT22 cells upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. Protein expressions were quantified. Sev: Sevoflurane; AA: Alisol A; PI: Propyl iodide; FITC: Fluorescein isothiocyanate; PARP: poly ADP-ribose polymerase.

3.3 Alisol A alleviates Sev-induced ferroptosis

Alisol A effects on Sev-induced HT22 cell ferroptosis were detected. DCF staining showed that Sev treatment increased DCF staining of HT22 cells, suggesting oxidative stress promotion (Fig. 3A). However, Alisol A further decreased DCF staining intensity in Sev-induced HT22 cells (Fig. 3A). Further, Sev increased MDA and decreased GSH levels in HT22 cells. Alisol A treatment, however, reversed the levels of these factors caused by Sev treatment in HT22 cells (Fig. 3B). Similarly, Alisol A treatment reversed Fe^{2+} levels in HT22 cells caused by Sev treatment (Fig. 3C). According to Immunoblot analysis, HT22 cells treated with Sev displayed decreased expression of GPX4 and SLC7A11, while Alisol A increased these expressions, suggesting ferroptosis promotion (Fig. 3D). Therefore, Alisol A alleviates Sev-induced ferroptosis.

3.4 Alisol A activates SIRT3 in Sev-induced HT22 cells

Finally, a possible mechanism was explored. qPCR and Immunoblot assays indicated that Sev treatment suppressed SIRT3 expression in HT22 cells (Fig. 4A,B). However, Alisol A contributed to SIRT3 expression in Sev-induced HT22 cells (Fig. 4A). An inhibitor of SIRT3, 3-TYP was then used to suppress its activity. Immunoblot assays showed that Sev treatment suppressed HT22 cell growth, whereas Alisol A decreased Sev-induced HT22 cell growth (Fig. 4C). However, 3-TYP further decreased Sev-induced HT22 cell growth after Alisol A treatment (Fig. 4C). Similarly, immunoblot assays indicated that 3-TYP further increased expression of cleaved caspase 3 and cleaved PARP in Sev-induced HT22 cells upon Alisol A treatment, suggesting apoptosis inhibition (Fig. 4D,E). Immunoblot assays revealed that Alisol A treatment reversed suppression of GPX4 and SLC7A11 in Sev-induced HT22 cells, whereas 3-TYP further decreased these expressions, suggesting ferroptosis suppression (Fig. 4F). In general, Alisol A activates SIRT3 in Sev-induced HT22 cells.

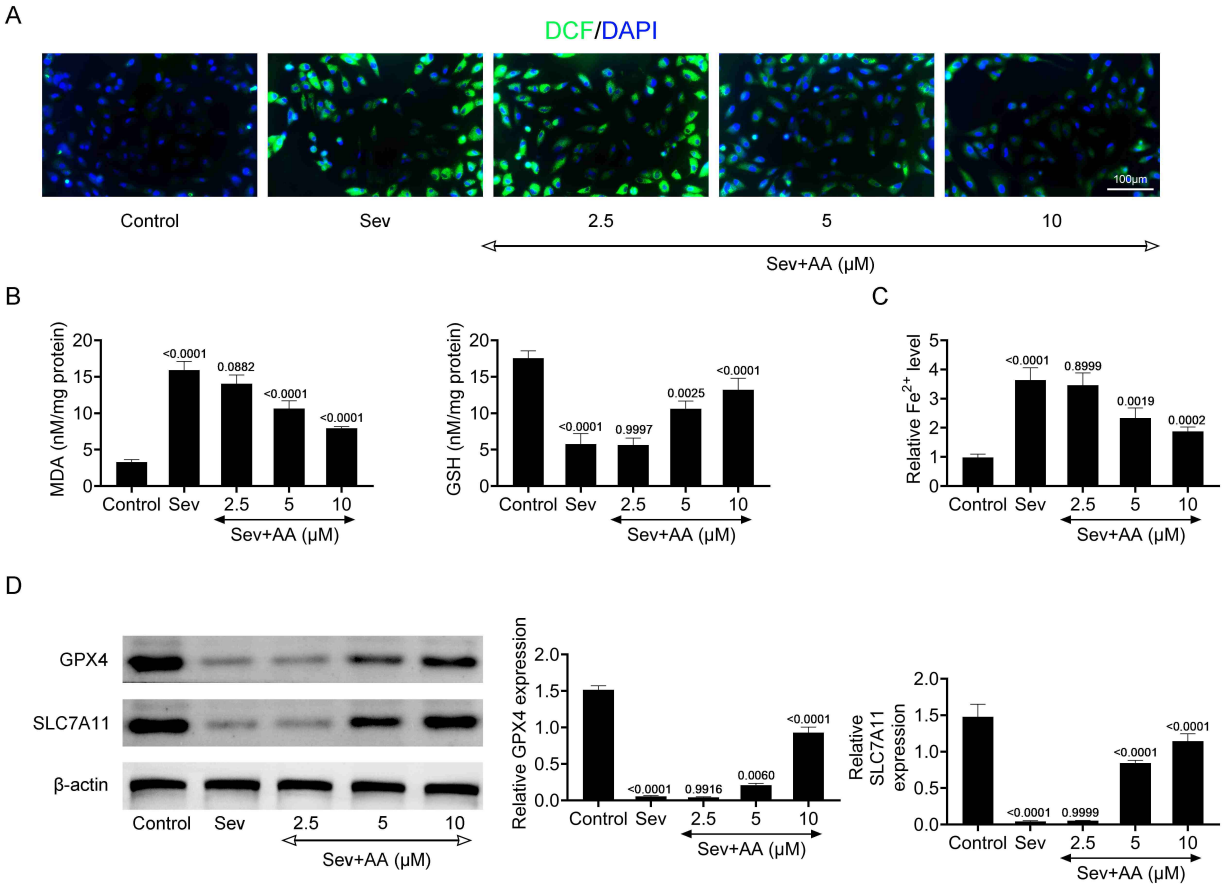

FIGURE 3. Alisol A alleviates Sev-induced ferroptosis. (A) DCF staining showed HT22 cell oxidative stress upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. (B) The indicated kits revealed MDA and GSH levels in HT22 cells upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. (C) The Fe^{2+} detection kit showed the Fe^{2+} levels in HT22 cells after Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. (D) Immunoblot assays showed GPX4 and SLC7A11 expressions in HT22 cells upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. Protein expressions were quantified. Sev: Sevoflurane; AA: Alisol A; DCF: 2′,7′-Dichlorofluorescein; DAPI: 4,6-diamino-2-phenyl indole; MDA: Malondialdehyde; GPX4: glutathione peroxidase 4; SLC7A11: Solute Carrier Family 7 Member 11.

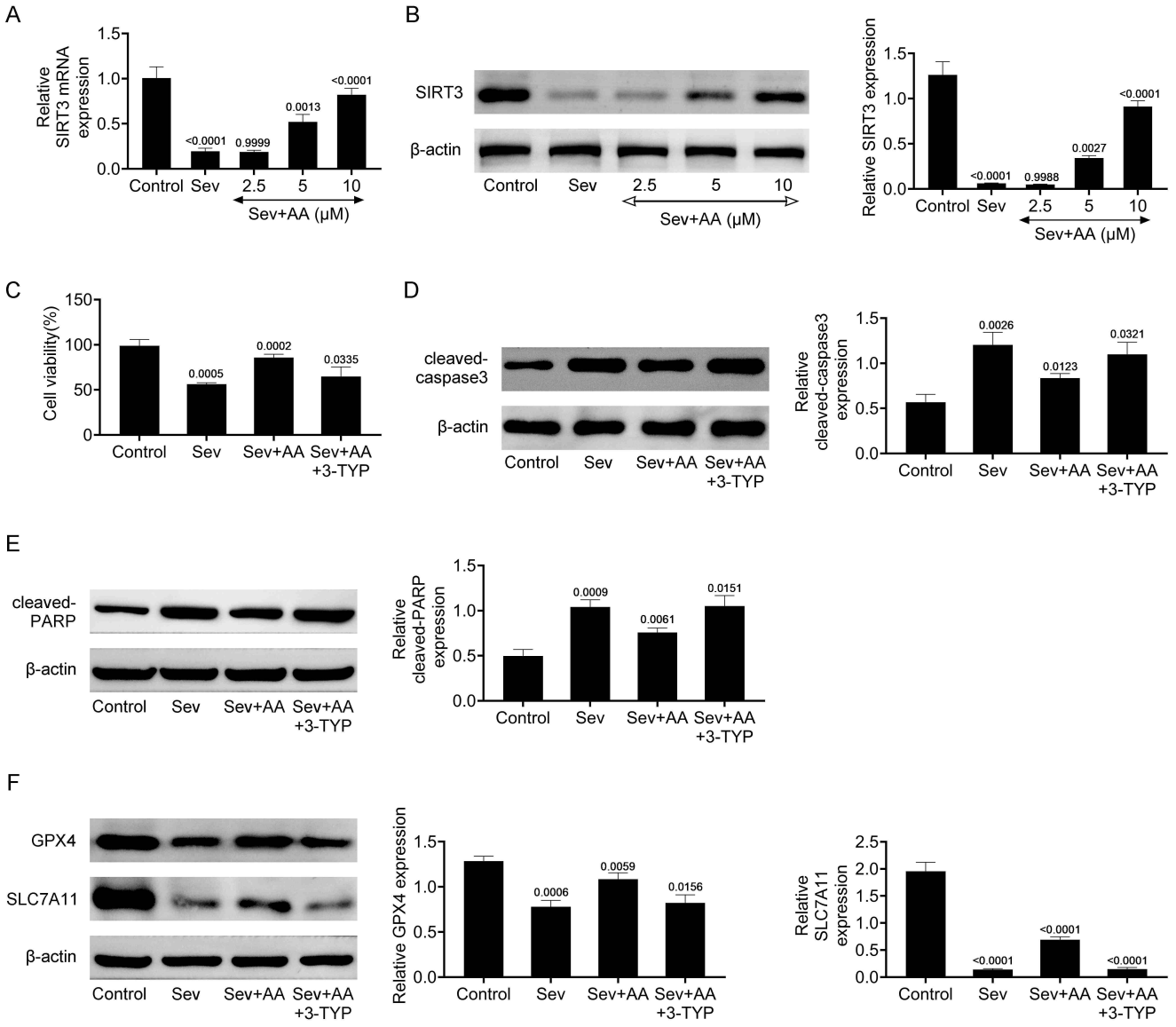

FIGURE 4. Alisol A activates SIRT3 in Sev-induced HT22 cells. (A) qPCR assays examined SIRT3's mRNA levels in HT22 cells upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. SIRT3 relative expression was quantified. (B) Immunoblot assays revealed SIRT3 expression in HT22 cells upon Sev and AA treatment at 0, 2.5, 5 and 10 μM for 24 h. SIRT3 relative expression quantified. (C) Alisol A's effects on HT22 cell growth were evaluated by CCK-8 assays after Sev, AA and 3-TYP treatment for 24 h. OD450 value was measured. (D) Immunoblot assays showed cleaved caspase 3 expression in HT22 cells after AA and 3-TYP treatment for 24 h. SIRT3 relative expression was quantified. (E) Immunoblot assays revealed cleaved PARP expression in HT22 cells upon AA and 3-TYP treatment for 24 h. SIRT3 relative expression was quantified. (F) Immunoblot assays revealed GPX4 and SLC7A11 expressions in HT22 cells upon AA and 3-TYP treatment for 24 h. Relative expressions of GPX4, and SLC7A11 were quantified. Sev: Sevoflurane; AA: Alisol A; SIRT3: Sirtuin 3; 3-TYP: 3-(Benzylamino)-3-Methylbutanoic Acid; PARP: poly ADP-ribose polymerase; GPX4: glutathione peroxidase 4; SLC7A11: Solute Carrier Family 7 Member 11.

4. Discussion

Sev, an inhalational anesthetic, is commonly used in surgery. It has been associated with neurotoxic effects, particularly in developing brains. Prolonged or repeated exposure to Sev may cause cognitive deficits and behavioral changes in children. Sev-induced neurotoxicity may result from inflammation, oxidative stress and neuronal apoptosis, but the exact mechanisms are unclear. There is evidence that Sev activates nuclear factor (NF)-κB, a transcription factor involved in inflammatory responses [11]. Consequently, Sev may cause neuroinflammation, which could have implications for its neurotoxic effects, particularly in developing brains. Sev and neuroinflammation are still under investigation, emphasizes the importance of exploring potential inflammatory responses induced by Sev in neurological settings. This study indicated that Alisol A attenuates Sev-induced cell damage in neurons. Alisol A may therefore be an effective treatment for Sev-induced neurotoxicity.

Ferroptosis-related neurotoxicity is a phenomenon where

excessive iron levels in the brain can lead to oxidative stress, inflammation and neuronal damage, potentially contributing to neurodegenerative diseases [12]. Ferroptosis promotes free radicals, causing cellular damage and neuroinflammation. Iron homeostasis within cells is maintained by ferritin, a key iron storage protein. Ferritin and iron dysregulation can cause oxidative stress and ferroptosis, resulting in neuroinflammation and neuronal damage. Strategies to mitigate ferroptosis-induced neurotoxicity may impact neurodegenerative prevention or management. The aim of ongoing research is to identify the precise mechanisms and possible therapeutic interventions. In this study, Alisol A reduces Sev-induced ferroptosis. Therefore, it could serve as a drug for Sev-induced neurotoxicity.

Alisol A is a natural compound found in the Alisma orientalis plant with various pharmacological properties. It has demonstrated anti-inflammatory, anti-cancer, hepatoprotective, and anti-diabetic properties. Among its mechanisms of action, Alisol A inhibits inflammatory pathways, inhibits cancer cell proliferation, induces apoptosis, and regulates metabolic pathways [10, 13, 14]. This study confirmed its protective effects on Sev-induced neurotoxicity. However, further research is needed to determine the exact mechanism.

SIRT3, a mitochondrial deacetylase enzyme, plays a crucial role in the nervous system by regulating various processes. It maintains mitochondrial function, reduces oxidative stress, and promoting neuronal survival. In the nervous system, SIRT3 prevents neurodegeneration, modulates synaptic plasticity, and influences neuronal metabolism. The fact that it plays a role in maintaining neuronal health makes it a potential therapeutic target in in neurodegenerative diseases and neurotoxicity-related conditions. SIRT3 upregulation mitigates neurotoxicity by promoting mitochondrial function and regulating antioxidant defenses [15–17]. Since SIRT3 maintains neuronal health, it may be a potential therapeutic target for neurotoxicity-related conditions [18]. Furthermore, this study demonstrated that Alisol A inhibits Sev-induced HT22 cell damage by activating SIRT3, further proving that SIRT3 may be a promising target in this disease.

Besides SIRT3 activation, further exploration of other signaling pathways are needed to fully elucidate the mechanisms by which Alisol A exerts its neuroprotective effects. Potential pathways of interest include the AMP-activated protein kinase (AMPK), Phosphatidylinositol 3-kinase (PI3K)/Serine/threonine protein kinase B (Akt), and NF-κB pathways, which play significant roles in neuroinflammation and neuronal survival. It may be possible to discover additional therapeutic targets for reducing Sev-induced neurotoxicity by exploring these pathways and understanding Alisol A's multifaceted actions.

This study is limited by the lack of *in vivo* research. A mouse model of Sev neuroinflammation will be constructed, Alisol A's role in it will be investigated. Multi omics analysis is necessary for further revealing potential downstream targets' regulatory mechanisms.

5. Conclusions

In summary, Alisol A inhibits Sev-induced HT22 cell damage by activating SIRT3. Alisol A may therefore be a promising drug for treating Sev-induced neuron damage.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

JH—designed the study and carried them out. JH and TYL—supervised the data collection, analyzed the data, interpreted the data. JH and MZ—prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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