

ORIGINAL RESEARCH

Moscatalin inhibits sevoflurane-induced inflammatory and oxidative stress injury in hippocampal neuronal HT22 cells

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Abstract

In order to analyze the effect and mechanism of Moscatilin on sevoflurane-induced HT22 hippocampal neuron cell (HT22) injury, sevoflurane was applied to HT22 cells to create an *in vitro* model of nerve damage, and Moscatilin was given to observe its effect on indicators of nerve injury. The effect of Moscatilin on sevoflurane-induced cell injury was measured based on cell viability (Cell Counting Kit-8), the expression of inflammatory factors interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in the cells was detected by Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR). Reactive oxygen species (ROS) production in the neuronal cells was detected by oxidative stress measurements based on the 2',7'-Dichlorofluorescein (DCF) assay. Using flow cytometry, the rate of apoptosis was determined, and the expression of apoptosis-related markers cleaved-caspase3 and cleaved-Poly Adp Ribose Polymerase (cleaved-PARP), and nuclear factor- κ -gene binding (NF- κ B)-related proteins were detected by western-blot analysis. The results showed that sevoflurane caused a significant decrease in HT22 cell viability, an increase in inflammatory factors TNF- α , IL-6 and IL-1 β , ROS and apoptosis, an increase in apoptosis-related markers, cleaved-caspase3 and cleaved-PARP, as well as in the p-p65/p65 ratio of NF- κ B-related proteins, and a decrease in the expression of inhibitor of NF- κ B α (I κ B α) protein. Moscatilin treatment counteracted sevoflurane-induced reduction in cell viability, decreased inflammatory factors and ROS production, inhibited sevoflurane-induced apoptosis, suppressed p-p65/p65 ratio, and increased I κ B α protein expression. In conclusion, Moscatilin inhibits the NF- κ B pathway and ameliorates sevoflurane-induced inflammatory responses, ROS production and apoptosis in HT22 hippocampal neuronal cells.

Keywords

Sevoflurane; Moscatilin; NF- κ B; ROS; Apoptosis

1. Introduction

Sevoflurane (Sev) is a fast-acting, fast-dissipating inhalant anesthetic widely used in the clinic. Nevertheless, inhalation of sevoflurane may induce cognitive dysfunction, leading to hippocampal neuronal apoptosis and neuroinflammation [1–3]. Treatment of HT22 hippocampal neuronal cells with sevoflurane resulted in a significant increase in inflammatory factor levels interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), and a dramatic decrease in antioxidant functions [4]. Therefore, the development of efficacious medications to mitigate sevoflurane-induced neuronal cell toxicity is imperative.

Numerous investigations have shown the ameliorative effects of various natural medicines on neurological disorders. Moscatilin is a bibenzyl polyphenol natural product isolated from *Dendrobium loddigesii*, and possesses a variety of phar-

macological properties, including anti-inflammatory, antioxidant and anti-tumor effects. Moscatilin was shown to inhibit lipopolysaccharide-induced NF- κ B activation in the AW264.7 macrophage cell line [5]. Moscatilin inhibits breast cancer proliferation, promotes breast cancer cell apoptosis, and has the potential to treat breast cancer therapeutically [6]. Moscatilin also inhibits the growth of human esophageal tumors and enhances the sensitivity of esophageal cancer cells to radiation [7]. In addition, Moscatilin has an ameliorative effect on neurologic injury disorders. Furthermore, Moscatilin was reported to improve learning and to counteract memory impairment in Alzheimer's disease [8].

NF- κ B plays a crucial role during inflammation and innate immune responses. When neural tissue is attacked by microbial pathogens, I κ B kinase (IKK) is activated, inducing I κ B phosphorylation. As a result, the NF- κ B dimer is dissociated and enters the nucleus to promote inflammatory factors,

including IL-6 and TNF- α inflammatory gene transcription, thereby activating inflammatory responses in the neural tissue [9].

In this study, an *in vitro* model was established by treating HT22 hippocampal neuronal cells with Sevoflurane, to investigate the effects of Moscatilin on Sevoflurane-induced neuronal cell damage, including inflammation, oxidative stress and apoptosis. It is hoped that this study will identify potential therapeutic agents for sevoflurane-induced neuronal cell damage.

2. Methods

2.1 Cell culture and treatment

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium, containing 10% fetal bovine serum and 1% penicillin/streptomycin, was used to cultivate mouse hippocampus neuronal cells HT22 (American Type Culture Collection). For Sevoflurane exposure, cell culture plates were placed in a closed chamber and cells were treated with 4% Sevoflurane for 6 hours. For Moscatilin treatment, the cells were incubated with distinct concentrations of Moscatilin for 24 hours before adding Sevoflurane, and cells in the control group were left untreated.

2.2 Cell viability assay

Cell viability in each cell treatment group was assessed using the Cell Counting Kit-8 (CCK-8). HT22 cells were inoculated into 96-well plates at a 5×10^3 cells/well density, and 10 μ L of CCK-8 solution was applied to each well. An enzyme marker was used to quantify absorbance at 450 nm nanometre. The results are presented as the percentage of viable cells in Moscatilin treatment versus control groups.

2.3 Measurement of TNF- α , IL-1 β and IL-6 production by enzyme-linked immunosorbent assay

To measure production of TNF- α , IL-1 β and IL-6 by the distinctly treated cell groups, the ELISA kit instructions were followed to measure levels of TNF- α (Elabscience, China, Wuhan, E-EL-M3063), IL-1 β (Elabscience, E-EL-M0037c) and IL-6 (Elabscience, E-EL-M0044c).

2.4 Detection of reactive oxygen species (ROS)

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is a cell membrane permeable and non-fluorescent compound. After entering the cell, DCFH-DA is hydrolyzed by cell esterase to generate 2',7'-dichlorodihydrofluorescein (DCFH), which is then quickly oxidized to produce the potent luminous products 2',7'-Dichlorofluorescein (DCF) due to the presence of reactive oxygen species.

2.5 RT-qPCR

PrimeScriptTM RT kit was utilized to reverse transcribe the isolated total RNA into complementary DNA (cDNA)

after the cells were treated with Trizol reagent. Real-time quantitative PCR was carried out using a CFX96 real-time system and SYBR Green I [10]. The messenger RNA (mRNA) levels of inflammatory factors TNF- α , IL-1 β and IL-6 were determined using β -actin as an internal reference. The primer sequences were as follows: IL-6: Forward TTCTGGGACTGATGCTG, Reverse CTGGCTTTGTCTTTCTTGTT; IL-1 β : Forward AGGCTCCGAGATGAACAA, Reverse AAGGCATTAGAAACAGTCC; β -actin: Forward CTCATGAAGATCCTGACCGAG, Reverse AGTCTAGAGCAACATAGCACAG.

2.6 Detection of cell apoptosis by flow cytometry

To find out the HT22 cell apoptosis rate, the Annexin V-Fluorescein Isothiocyanate/Propidium apoptosis detection kit was utilized. The cells were resuspended and stained with 5 μ L of Annexin V-Fluorescein Isothiocyanate (FITC) and Propidium (PI) in the dark. Subsequently, using flow cytometry, the apoptotic cell apoptosis rate was examined.

2.7 Western-blot

Radio-Immunoprecipitation Assay lysate was used to lyse the cells while they were chilled, and the total proteins in the cells were extracted by Bicinchoninic acid disodium salt kit. Then the proteins in the samples were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel electrophoresis system, and the protein bands were transferred to polyvinylidene fluoride (PVDF) membranes, and the 5% containment solution was prepared by adding TBS + Tween (TBST) solution to skimmed milk powder, and the PVDF membranes were incubated in the containment solution for 1 h in order to avoid the non-specificity of antibody-antibody binding. After washing, the PVDF membrane was incubated with the primary antibody in a refrigerator at 4 °C. The PVDF membrane incubated with the secondary antibody. Finally, the bands were color-developed by Enhanced chemiluminescence solution, and the protein was quantified using ImageJ software [11]. Antibody information was as follows: cleaved-caspase3 (Cell signaling, #9661, Danvers, MA, USA); cleaved-Poly Adp Ribose Polymerase (PARP) (Cell signaling, #9541); p-p65 (Cell signaling, #3033); p65 (Cell signaling, #8242); p-I κ B α (Cell signaling, #2859); I κ B α (Cell signaling, #4812); β -actin (Cell signaling, #4967).

2.8 Statistical analysis

Independent Student's *t*-tests were used to evaluate differences between two groups, while one-way Analysis of Variance and Tukey *post hoc* tests were used to evaluate differences between multiple groups. $p < 0.05$ was deemed significant. All experiments were repeated three times independently.

3. Results

3.1 Effect of moscatilin on sevoflurane-induced impact on HT22 cell viability

To test the cytotoxicity of Moscatilin (Fig. 1A), the cells were treated with different concentrations of Moscatilin, and then cell viability was detected, and it was found that 0.1, 0.5 and 1 $\mu\text{mol/L}$ of Moscatilin had no effect on the cell viability, and 2 $\mu\text{mol/L}$ of Moscatilin significantly decreased the cell viability (Fig. 1B). To verify the effect of Moscatilin on cell viability induced by Sev, the cells were treated with sevoflurane and 0.1, 0.5 and 1 $\mu\text{mol/L}$ Moscatilin, and it was found that Sev significantly decreased cell viability, whereas 0.1, 0.5 and 1 $\mu\text{mol/L}$ Moscatilin significantly increased cell viability induced by Sev (Fig. 1C).

3.2 Moscatilin attenuates sevoflurane-induced cellular inflammation

To explore the effects of Moscatilin on cellular inflammatory responses induced by sevoflurane, IL-1 β , IL-6 and TNF- α production and mRNA levels were measured by ELISA and qPCR. It was found that sevoflurane significantly increased the cellular levels of IL-1 β , IL-6, TNF- α content (Fig. 2A) and mRNA expression (Fig. 2B). Importantly, Moscatilin treatment significantly decreased the sevoflurane-induced increase in these inflammatory factors.

3.3 Moscatilin attenuates sevoflurane-induced production of reactive oxygen species (ROS)

To investigate effects of Moscatilin on sevoflurane-induced ROS production as a measure of a proinflammatory response, ROS production was quantified based on the DCF method. The results indicate that sevoflurane significantly increased ROS production, and that Moscatilin counteracted the sevoflurane-induced generation of ROS in HT22 hippocampal neuronal cells (Fig. 3).

3.4 Moscatilin attenuates sevoflurane-induced apoptosis

To help understand the cytoprotective effects of Moscatilin upon sevoflurane exposure, induction of apoptosis was measured in sevoflurane exposed HT22 cells, with and without Moscatilin treatment. The effect of Moscatilin on sevoflurane-induced apoptosis was examined by flow cytometry by measuring exposure of phosphatidyl serine by dying cells based on Annexin V binding, cleaved-caspase3, and cleaved-PARP. The results showed that sevoflurane significantly increased the rate of apoptosis (Fig. 4A), along with the expression of apoptosis-associated proteins cleaved-caspase3 and cleaved-PARP (Fig. 4B). Importantly, Moscatilin decreased the rate of apoptosis induced by sevoflurane exposure. This was documented based on the reduction of apoptosis-associated protein expression exemplified by cleaved-caspase3 and cleaved-PARP. Thus, the results indicate that Moscatilin inhibited sevoflurane-induced apoptosis.

3.5 Moscatilin inhibits the sevoflurane-induced NF- κ B pathway activation

To explore the mechanism by which Moscatilin ameliorates sevoflurane-induced neuronal cell damage, the relative expression of phosphorylated-p65 (p-p65), p65, p-I κ B α and I κ B α proteins was determined by quantitative western-blot analysis. The results showed that sevoflurane increased the expression of p-p65/p65 and p-I κ B α proteins, and decreased the expression of I κ B α proteins, thereby indicating activation of NF- κ B pathway activation in HT22 cells through sevoflurane exposure. Importantly, this cellular reaction to sevoflurane was counteracted by Moscatilin treatment as Moscatilin decreased the expression of p-p65/p65 and p-I κ B α proteins, and increased the expression of I κ B α proteins, these results suggest that Moscatilin inhibits sevoflurane-induced NF- κ B pathway activation (Fig. 5).

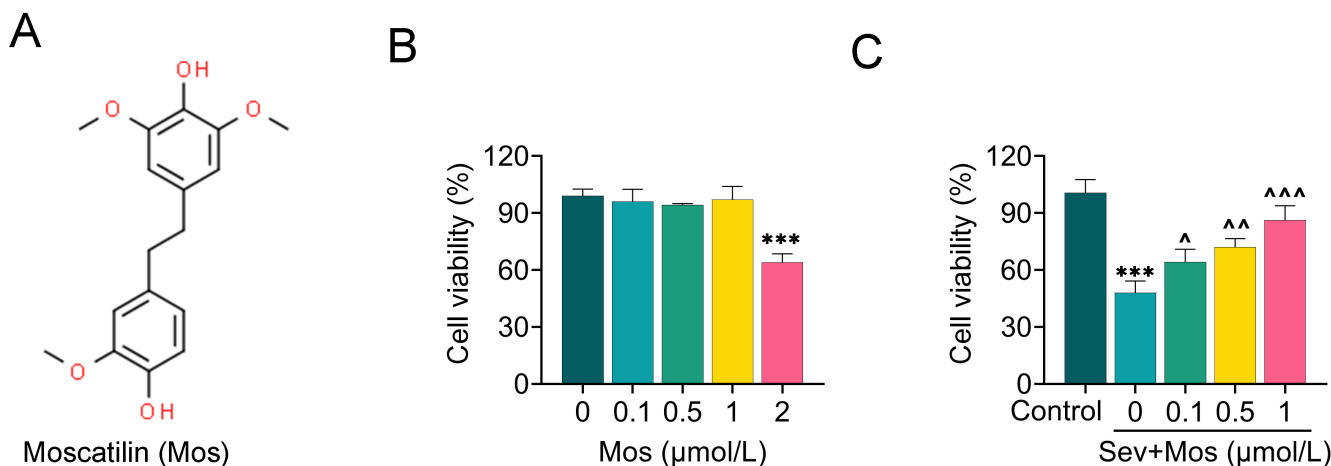
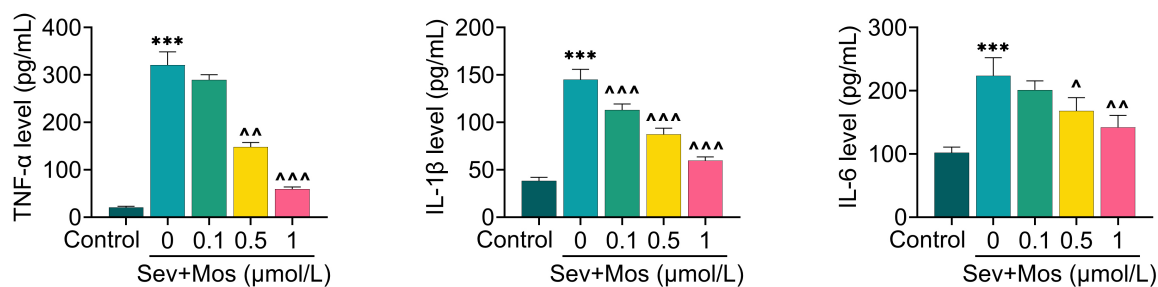


FIGURE 1. Effect of Moscatilin on HT22 cell viability. (A) Chemical structural formula of Moscatilin. (B) The CCK-8 cell count assay detects the effect of different concentrations of Moscatilin on cell viability. $p < 0.001$ (vs. 0 $\mu\text{mol/L}$). (C) Effect of CCK-8 detection of Moscatilin on sevoflurane-induced cell viability. *** $p < 0.001$ (vs. the control group); ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ (vs. the sevoflurane only group).

A



B

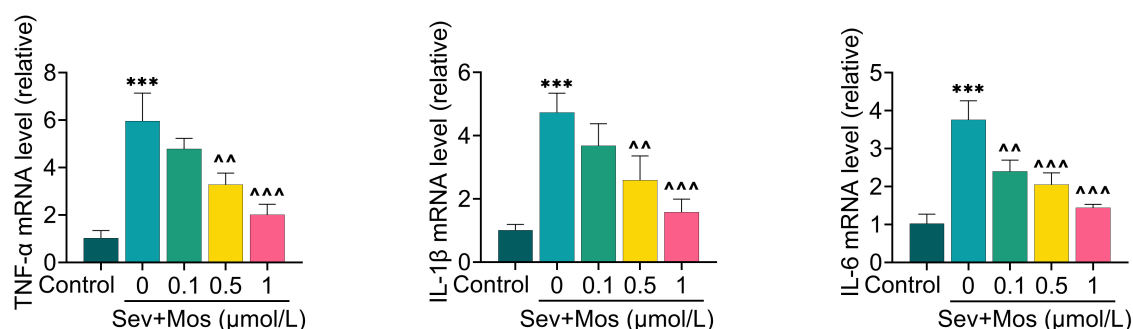


FIGURE 2. Moscatilin attenuates sevoflurane-induced inflammatory cell responses. (A) Effect of Moscatilin on sevoflurane-induced cellular inflammatory factors TNF- α , IL-6 and IL-1 β detected by ELISA. (B) Effect of Moscatilin on sevoflurane-induced cellular inflammatory factors TNF- α , IL-6 and IL-1 β mRNA by PCR. *** $p < 0.001$ (vs. the control group); ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ (vs. the Sev group). TNF: tumor necrosis factor; IL: interleukin.

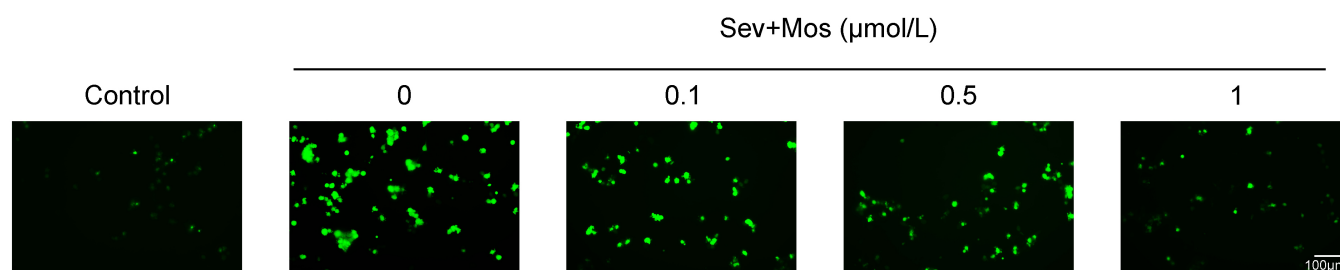


FIGURE 3. Moscatilin attenuates sevoflurane-induced ROS production. Effect of DCF assay of Moscatilin on sevoflurane-induced ROS.

4. Discussion

Sevoflurane is a volatile compound and widely used as an inhalant anesthetic in clinical practice. Improper use has been reported to cause neuronal cell damage, including cognitive dysfunction, neuronal cell apoptosis, inflammation [12] and oxidative stress [13]. In this study, the effects of Moscatilin on murine hippocampal neuronal cells exposed to sevoflurane were investigated, to explore whether Moscatilin might protect neuronal cells from sevoflurane-induced damage. Our results showed that Moscatilin can inhibit the NF- κ B pathway and prevent sevoflurane-induced neuronal cell apoptosis, oxidative stress and inflammation, thereby potentially protecting nerves from damage by sevoflurane use. Thus, our

study identifies Moscatilin as a potential therapeutic agent to counteract sevoflurane-induced nerve injury.

Previous studies have confirmed that Moscatilin ameliorates nerve damage and that Moscatilin prevents diabetic neuropathy by inhibiting methylglyoxal-induced cytotoxicity in a concentration-dependent manner, through inhibition of ROS [14]. Consistent with the above studies, in our present study, we confirmed that Moscatilin inhibited sevoflurane-induced HT22 hippocampal neuronal cytotoxicity and suppressed ROS production. Sevoflurane administration leads to increased expression of inflammatory cytokines (TNF- α , IL-6 and IL-1 β), inflammatory cytokine stimulation leads to activation of the NF- κ B pathway, and activation of the NF- κ B pathway continues to stimulate the transcription of inflammatory

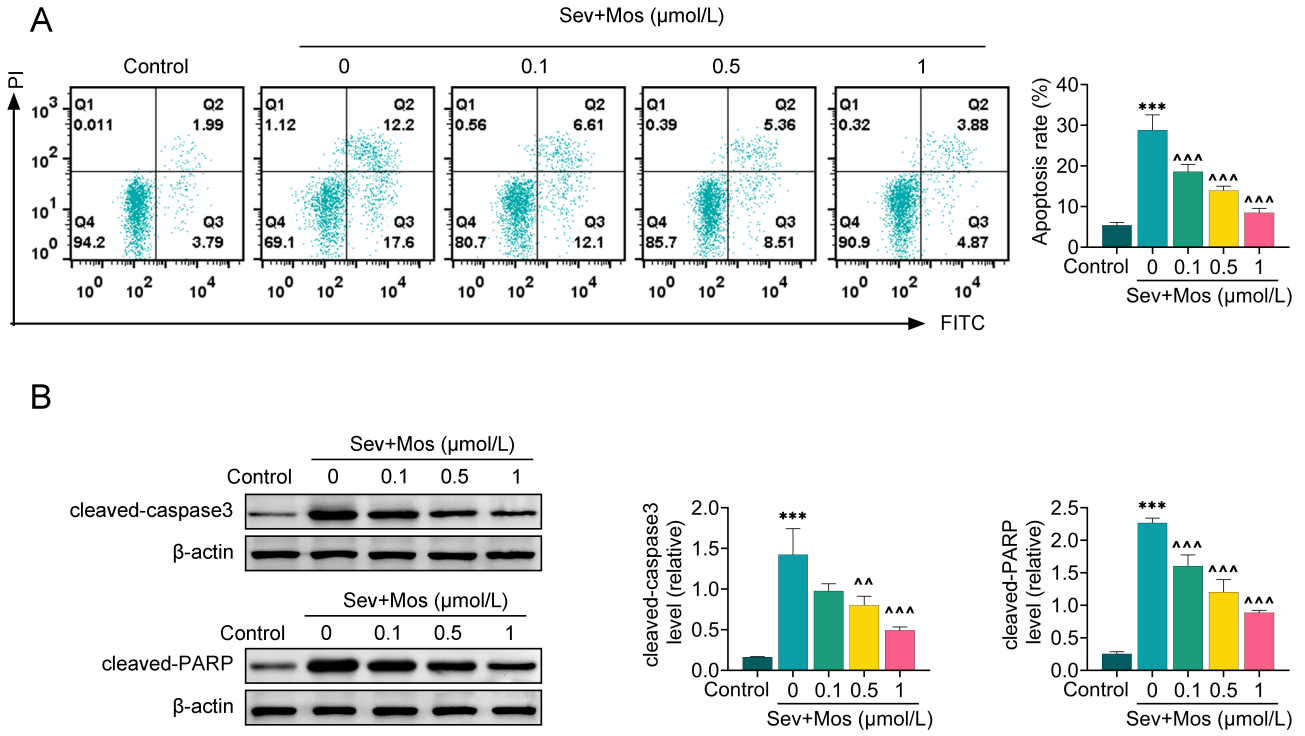


FIGURE 4. Moscatilin attenuates sevoflurane-induced apoptosis. (A) Effect of Moscatilin on the rate of apoptosis induced by sevoflurane, detected by flow cytometry. (B) Effect of Moscatilin on sevoflurane-induced expression of apoptosis markers cleaved-caspase3 and cleaved-PARP detected by western-blotting. $***p < 0.001$ (vs. the control group); $^{\wedge}p < 0.01$, $^{\wedge\wedge}p < 0.001$ (vs. the sevoflurane only group). PI: Propidium; FITC: Fluorescein Isothiocyanate; PARP: poly ADP-ribose polymerase.

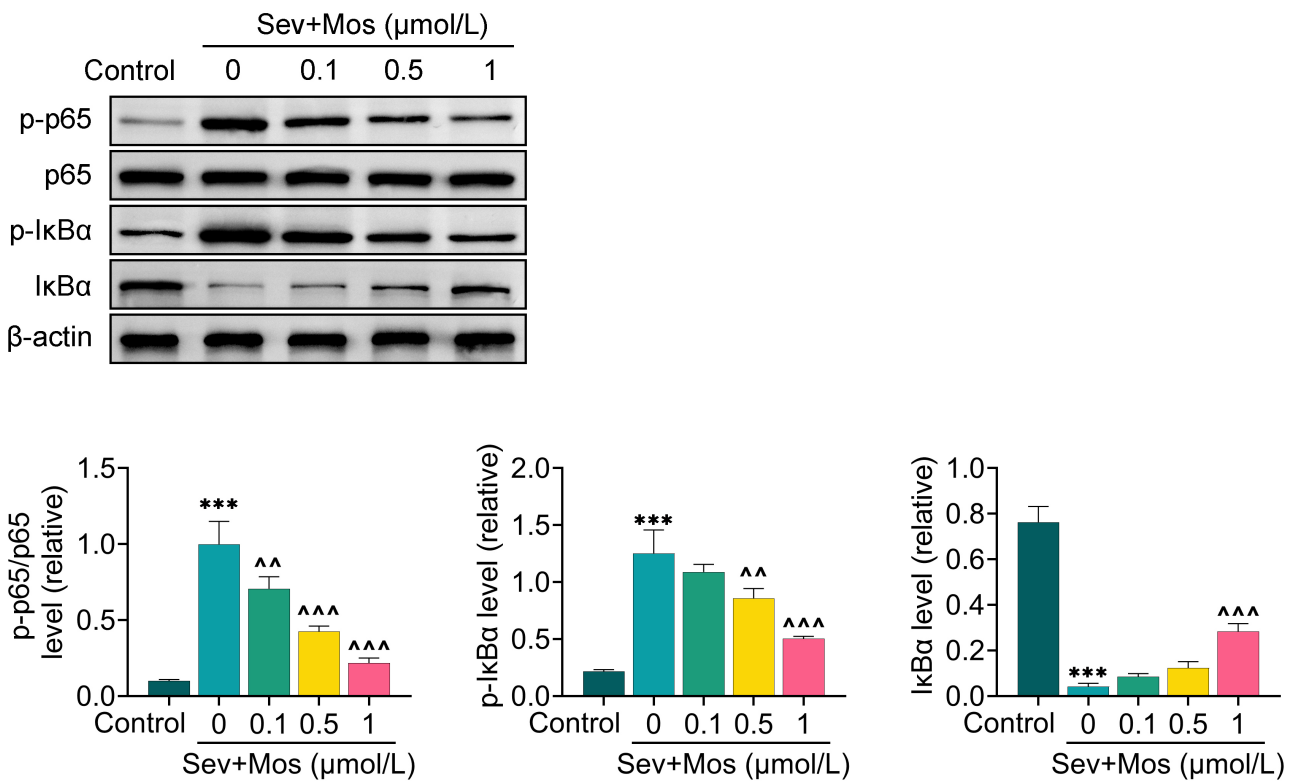


FIGURE 5. Moscatilin inhibits sevoflurane-induced NF-κB pathway activation. Effect of Moscatilin on sevoflurane-induced protein expression of p-p65, p65, p-IκBα and IκBα detected by western-blot analysis. $***p < 0.001$ (vs. the control group); $^{\wedge}p < 0.01$, $^{\wedge\wedge}p < 0.001$ (vs. the Sev group).

factors, resulting in a vicious cycle. Sevoflurane also increases the expression of key proteins in the apoptotic process, including cleaved-caspase-3 and cleaved-PARP, while the stimulation of inflammatory factors and ROS production further stimulate apoptosis [15].

Recently, an increasing number of bioactive compounds have been shown to exert neuroprotective effects by inhibiting the NF- κ B pathway. For example, resveratrol pretreatment ameliorated sevoflurane-induced elevation of IL-6 and TNF- α levels, and inhibited the NF- κ B pathway [16]. Carnosol attenuated sevoflurane-induced neuroinflammation and neuronal apoptosis by mediating the NF- κ B pathway [17]. These findings suggest that the NF- κ B signaling pathway may play an important role in sevoflurane-induced inflammation and cell injury and is therefore a therapeutic target for alleviating the unwanted side effects of sevoflurane exposure. It was previously shown that Moscatilin ameliorated the loss of neuronal cell viability and ROS production induced by late glycosylation end products, decreased the expression of the pro-apoptotic key enzymes cleaved-Caspase3 and cleaved-PARP, inhibited the p-p65/p65 ratio, and increased I κ B α [18]. Consistent with the findings described above, in our present study Moscatilin inhibited sevoflurane-induced inflammatory factor production and apoptosis, suppressed the p-p65/p65 ratio, and increased I κ B α protein expression, confirming that Moscatilin inhibits the NF- κ B pathway and ameliorates sevoflurane-induced neuronal cell injury.

5. Conclusions

This study established a model of sevoflurane-induced neurological injury using the murine HT22 hippocampal neuronal cell line. Leveraging this model, we found that Moscatilin was able to inhibit the NF- κ B pathway activity and exert protective effects such as anti-inflammatory, anti-ROS, and anti-neuronal apoptosis functions. Therefore, Moscatilin may be a potential therapeutic drug to counteract sevoflurane-induced neurological injury. However, there are limitations in this study, due to the limited experimental conditions, no animal *in vivo* model was established to evaluate the effect of Moscatilin *in vivo*, and the *in vivo* experiments will be continued in future studies.

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

LLZ and YHZ—designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared 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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