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Original article

Hispidulin prevents sevoflurane— Induced memory dysfunction in aged rats

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ABSTRACT

Aim: As a widely used general anesthetic, sevoflurane has been found to induce cognitive and memory defectsin the elderly. This may increase the risk of Alzheimer's disease. This study explores the neuroprotective effect of hispidulin, a natural flavone compound, against sevoflurane-induced memory dysfunction.

Methods: The effect of sevoflurane exposure on memory function was evaluated by novel object recognition and Y-maze testing using an aged rat model. The apoptotic cell death in the hippocampus of rats was assessed using a TUNEL assay. The levels of protein markers for cell apoptosis in the hippocampus were examined by western blot. The effect of sevoflurane and hispidulin on the accumulation of Aβ was also examined. In addition, the attenuating effect of hispidulin on sevoflurane-induced neuroinflammation was assessed by measuring the expression of pro-inflammatory cytokines and the translocation of NF-κB p65 from cytosol to nucleus. The activation of Nrf2 in the hippocampus was also detected. Moreover, the effect of hispidulin on sevoflurane-induced apoptosis, Aβ accumulation, and neuroinflammation was also examined in human neuroglioma H4 cells, which served as an *in vitro* model.To further examine the role of Nrf2 in the neuroprotective activity of hispidulin against sevoflurane-neurotoxicity, H4 cells were transfected with Nrf2 targeting siRNA, which led to a significant reduction in Nrf2 expression.

Results: Both novel object recognition and Y-maze testing showed that sevoflurane significantly impaired the memory of aged rats, which was significantly reversed by pretreatment with hispidulin. Mechanistically, our findings revealed that hispidulin significantly attenuated sevoflurane-induced apoptotic cell death, Aβ accumulation, and neuroinflammation. In agreement with *in vivo* studies, hispidulin was also able to attenuate sevoflurane-induced apoptosis, increases of Aβ levels, and neuroinflammation in H4 cells. Moreover, our results showed that Nrf2 activation mediated the neuroprotective effect of hispidulin against sevoflurane-induced neurotoxicity by demonstrating that knockdown of Nrf2 in H4 cells significantly compromised its protective effects.

Conclusion: As our study provides *in vitro* and *in vivo* evidence that hispidulin can offer protection against sevoflurane-induced neurological dysfunction, hispidulin has the potential to be a neuroprotective agent that can improve the cognitive and memory function of elderly patients undergoing anesthesia.

1. Introduction

Postoperative neurocognitive disorders, including postoperative delirium and postoperative cognitive dysfunction (POCD), refer to a clinical condition featured by cognitive dysfunction, lack of concentration, and impairment in ability to process information following surgery [1]. The prevalence of POCD in elderly populations (≥60 years old)is higher than that of young adults. About half of all elderly patients presented with POCD at the time of discharge, after which about 10% of elderly patients will exhibit persistent POCD for three months or longer [2]. For the majority of patients, POCD is a self-limiting condition that can spontaneously improve. However, POCD can increase morbidity and mortality, particularly in elderly patients [3]. For instance, POCD has been identified as a risk factor for Alzheimer's disease in elderly patients [4]. Although the factors that contribute to POCD have not been fully understood, several studies have established the association between the use of general anesthetics such as sevoflurane and the onset and progression of Alzheimer's in elderly patients. Given the larger proportion of elderly in our population, it is imperative to discover agents that can prevent sevoflurane-induced neurotoxicity and reduce the incidence of POCD.

Flavonoid compounds encompass more than 4000 polyphenolic compounds, and are commonly found in plants, including fruits, vegetables, and flowers [5]. Flavonoid compounds are categorized in to

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the following four main classes based on chemical structures: flavones, flavanones, catechins, and anthocyanins [6]. Interestingly, accumulating evidence has linked the intake of flavonoids with beneficial effects on brain function, especially in elderly populations [7,8]. For instance, elderly people who consume chronic flavanone-rich orange juice have shown improvements in cognitive function [9]. Hispidulin has a chemical structure of 4′,5,7-trihydroxy-6-methoxyflavone, and can be found in different plant materials such as Saussurea in volucrata Kar. et Kir., and several Artemisia and Salvia species [10]. Several studies have proved its role as an antifungal, anti-inflammatory, antioxidant, anti-thrombosis, antineoplastic, and anti-osteoporotic agent [11–21]. As regards to its effect on the central nervous system, hispidulin has been found to cross the blood brain barrier and exert an anticonvulsant effect [22]. *In vitro* studies have also shown that hispidulin can activate human benzodiazepine (BZD) receptors [11]. Furthermore, the neuroprotective effects of hispidulin against neurotoxicity induced by bupivacaine has also been documented in *in vitro* studies [23]. Encouraged by the previous study, we hypothesize that hispidulin might provide protection against sevoflurane-induced neurotoxicity. The present study aimed to test this hypothesis using a rat and cellular model. Our results revealed that pretreatment with hispidulin significantly reversed sevoflurane-induced memory impairment in aged rats. Moreover, our study provided experimental evidence that hispidulin exerted neuroprotective effects by attenuating sevoflurane-induced apoptotic cell death, Aβ accumulation, and neuroinflammation through activating Nrf2 signaling.

2. Materials and methods

2.1. Animals and treatment

Male Sprague-Dawley rats aged 20 months were purchased from Shandong University Laboratory Animal Center. The experimental rats were kept under SPF conditions with 12 h light and dark cycles. Air conditioning was set at 23 °C for one week prior to the start of the experiment. Food and water was available *ad libitum*. The experimental protocol was approved by the Medical Ethics Committee of Ji'nan Central Hospital Affiliated to Shandong University.

A total of 32 rats were allocated into the following four experimental groups: Vehicle (V), sevoflurane (Sevo), hispidulin (His), and sevoflurane+ hispidulin (Sevo + His). The rats in the His and Sevo + His groups received sevoflurane in the environment containing 4% sevoflurane for 4 h. Hispidulin was given via intraperitoneal injection as a single dose of 40 mg/kg at 12 h before sevoflurane exposure(In our preliminary experiments, we have compared the effect of single dose and double dose with a dosing interval of 12 h, which did not reveal any significant difference.Therefore, we chose a single dose regimen here.). The physiological parameters were recorded 24 h post sevoflurane exposure. The experimental protocol is illustrated in Fig. 1A.The rats were sacrificed by decapitation at the end of experimentation as previously described [24].

2.2. Novel object recognition test

To evaluate memory performance, a novel object recognition test (NOR test) was conducted following the protocols described in previous studies [25]. An open field apparatus with a dimension of 60 × 60 × 30 cm was utilized as a chamber. The action of sniffing or touching an object with the nose was considered as exploration. The discrimination index was calculated using the formula: [time spent with novel object − time spent with familiar object]/[total time exploring both objects]. An increased preference for the novel object was interpreted as successful memory retention for the familiar object. An absence of any difference in the exploration of the two objects was interpreted as memory deficit.

2.3. Y-maze testing

Immediate spatial working memory was evaluated by documenting spontaneous alternation behavior in a Y-maze apparatus as previously described [26]. One day after the post NOR test, rats were allowed for habituation in the apparatus for half an hour before the test started. One rat was then placed at the end of one arm and allowed to freely explore the maze for 10 min. Consecutive entry into three arms in an alternative order (spontaneous alternation) was defined as a successive entry on overlapping triplet sets. The percentage of spontaneous alternations was calculated as the ratio of observed to possible spontaneous alternation.

2.4. TUNEL assay of apoptosis

Following the Y-maze test, apoptotic cell death in the hippo campus was assessed by a TUNEL assay. Briefly, the brain of the rats was removed, fixed with paraformaldehyde (4%) at 4 °C overnight, embedded in paraffin, and processed into 25 μm sections. The TUNEL assay was conducted utilizing a Cell Death Detection Kit (Roche Diagnostics Corp, Indianapolis, IN) following manufacturer protocols.

2.5. ELISA assay for MDA content in the hippocampus

Following sacrifice, the hippocampus of the rats was processed as previously described [25]. Protein content was determined by a BCA assay kit(Thermo Scientific, Shanghai, China). The content of MDA in the total protein was measured by a commercial ELISA kit (Abcam, Shanghai, China).

2.6. Cell culture

Human neuroglioma H4 cells were obtained from Cell Bank of The Chinese Academy of Sciences (Shanghai, China). All cells were cultured in a RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 1% penicillin/streptomycin (Thermo Fisher Scientific), 2 mM glutamine (Gibco, Grand Island, NY), and 10% fetal bovine serum(FBS; Invitrogen, Carlsbad, CA). The cells were placed in a humidified incubator at 37 °C with a 5% CO_2environment. Sevoflurane was dispersed in the atmosphere at a concentration of 4.1% using an anesthesia machine.

2.7. Western blot

Nuclear and cytoplasmic proteins were extracted from the hippo campus and cells using a commercial lysis buffer as previously described (Beyotime, Shanghai, China). Protein content was determined by a BCA assay kit (Thermo Scientific, Shanghai, China). Western blotting was performed following standard protocols. Proteins were detected with specific primary antibodies against cleaved caspase-3, Bcl-2, Bax, and Nrf2 from Abcam(Shanghai, China); primary antibodies against BACE, IL-6, IL-1β, and Lamin A from Santa Cruz (Santa Cruz, CA); primary antibodies against Aβ(1–42), IκBα, *p*-IκBα, and NF-κB from Cell Signaling (Danvers, MA); and primary antibodies against TNF-α and β-actin from Beyotime (Shanghai, China). The immunoblots were detected with HRP-conjugated secondary antibodies form Beyotime (Shanghai, China). An ECL system was used to perform the western blot analysis (Vector Laboratories, Burlingame,CA).

2.8. Quantitative real-time PCR (qRT-PCR) assays for nrf2

Total RNA extracted from hippocampus tissues with the Trizol reagent (Invitrogen) was reversely transcribed to cDNA using PrimeScript Master Mix (TaKaRa, Dalian, China). To detect the mRNA expression of Nrf2, qRT-PCR was performed using the SYBRRT-PCR Kit (Takara). The housekeeping gene GAPDH was used as the control for Nrf2 mRNA expression. The primers for Nrf2 and GAPDH (as the control) are listed