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Regular paper

Cucurbitacin E reduces the cognitive dysfunction induced by sevoflurane in rats by regulating NF-κB pathway

Shaohua Zheng[1#], Bowen Shi[2#], Xin Li[1], Hui Yuan[1] and Yinglu Feng[3]✉

[1]Department of Anesthesiology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, 710061, China; [2]Department of Anesthesiology, Xi'an International Medical Center, Xi'an, China; [3]Department of Anesthesiology, Union hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan430022, China

Background: Perioperative neurocognitive disorders (PND) occur frequently and refer to alterations in cognitive function after surgery, especially in elderly patients. PND is characterized as abnormalities of learning, memory, language, and emotions. Cucurbitacin E has been reported to possess various pharmacological properties, including anticancer, antiviral, and anti-inflammatory effects. In this study, we investigated whether cucurbitacin E could alleviate sevoflurane-induced cognitive dysfunction in rats. Methods: Sprague-Dawley male rats (~6 weeks old) were randomly assigned to three groups: the control group, the Sevoflurane group, and the Sevoflurane + Cucurbitacin E group. Subsequently, the cognitive dysfunction of the rats was evaluated through the morris water maze test. Hematoxylin and eosin (HE) staining was used to measure the pathological change in brain tissues. Enzyme-linked immunosorbent assay (ELISA) kits were used for determinations of S-100 calcium binding protein B (S-100β) and neuron-specific enolase (NSE) and cytokine. Cell apoptosis was analyzed by TdT-Mediated Nick-End Labeling (TUNEL) staining. Protein levels were confirmed by Western blotting. Results: Cucurbitacin E relieved brain injury in rats induced by sevoflurane. Cucurbitacin E alleviated sevoflurane-induced S-100β and NSE levels. Additionally, the Morris water maze task revealed that cucurbitacin E attenuated cognition impairment in sevoflurane-induced rats. Sevoflurane increased levels of IL-6, TNF-α and IL-1β levels, and decreased the level of IL-10. However, cucurbitacin E exhibited opposite effects on these cytokines, which were induced by sevoflurane. Furthermore, cucurbitacin E inhibited sevoflurane-induced neuron apoptosis and NF-κB pathway in rats. Conclusion: These findings indicate that cucurbitacin E can improve sevoflurane-induced cognitive dysfunction in rats by regulating NF-κB pathway, which provided a new strategy for PND treatment.

Keywords: PND, cucurbitacin E, cognitive dysfunction, sevoflurane, NF-κB pathway

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✉e-mail: ylfeng2930@163.com
#These authors contributed equally to the work.

Abbreviations: Bcl-2, B-cell lymphoma 2; HE, hematoxylin and eosin; ELISA, linked immunosorbent assay; IL-6, interleukin-6; IL-10, interleukin-10; IκBα, IkappaBalpha; NF-κB, nuclear factor κB; S-100β, S-100 calcium-binding protein B; NSE, neuron-specific enolase; PND, perioperative neurocognitive disorders; p-p65, phosphorylated-p65; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1beta; TUNEL, TdT-Mediated Nick-End Labeling

INTRODUCTION

Postoperative cognitive dysfunction is characterized by cognitive impairments in patients who underwent major surgery. Approximately 10% of surgical patients and about 40% of elderly patients(age > 65-year-old) and even older experience postoperative cognitive dysfunction (Dong *et al.*, 2018). Previously, all forms of impairment were called postoperative cognitive dysfunction. However, perioperative neurocognitive disorders (PND) have recently been used as an overarching term for cognitive impairment, which is identified in the perioperative period (Evered *et al.*, 2018). The incidence of PND was 50%~70% at one week after coronary artery bypass grafting surgery and was maintained at 30% after 6 months (Newman *et al.*, 2006). Patients with PND had a lower survival rate and an employment rate after cardiac surgery (Newman *et al.*, 2001; Phillips-Bute *et al.*, 2006). The pathogenesis of PND is multifaceted, including anesthesia, tissue damage, surgical stress, psychological stress. Therefore, it is necessary to explore the mechanism of PND after surgery.

Nuclear factor κB (NF-κB) is activated by many types of growth factors in neuroinflammatory diseases. Moreover, NF-κB also plays an important role in cell apoptotic cell death (Shabab *et al.*, 2017). NF-κB has been found to be activated in synapses in excitatory synaptic transmission responses and functions in processes including learning and memory in the mature nervous system (Schmeisser *et al.*, 2012). The apoptosis of damaged nerve cells was alleviated while NF-κB signaling pathway was suppressed, which caused an improvement in learning memory function (Gu *et al.*, 2015). Based on these findings, it is of great importance to investigate the exact effects of NF-κB signaling pathway on PND.

Cucurbitacin E, as a member of the cucurbitacin family, is a tetracyclic triterpenoid compound, which is extracted from Cucurbitaceae plants (Hsu *et al.*, 2014). Cucurbitacin E has been reported to possess various pharmacological properties, including anticancer, antiviral, and anti-inflammatory effects (Chanda *et al.*, 2020). Additionally, cucurbitacin E showed antiapoptotic properties in dopaminergic neurons (Arel-Dubeau *et al.*, 2014) and had neuroprotective effects on neuronal injury in microglia (Park *et al.*, 2015). Here, we investigate the effects of cucurbitacin E on sevoflurane-induced brain injury, neuroinflammation, nerve cell apoptosis, and cognitive dysfunction *in vivo*. We aimed to explore whether cucurbitacin E could function in PND.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (~6 weeks old) were divided into the following groups (n=6/group): the Control group, the Sevoflurane group (Sev) and the Sevoflurane + Cucurbitacin E group (Sev + CE) group. In the Sev group, rats were exposed to 2.5% sevoflurane at 600 μg/kg/min in air for six hours in an anesthesia agent evaporator chamber as described in previous research (Wang *et al.*, 2018). In the Sev + CE group, rats received sevoflurane and also were intraperitoneally injected with 5 mg/kg CE. In the Control group, rats received regular air alone for six hours. After the Morris water maze task, blood samples from rats were collected and stored at −20°C. Finally, rats were intraperitoneally injected with overdose anesthesia (800 mg/kg pentobarbital) (Zatroch *et al.*, 2017). After the rats were sacrificed, brain tissues were collected and used for the following experiments. All animal experiments in this study were carried out in accordance with the Guide for the Care and Use of the First Affiliated Hospital of Xi'an Jiaotong University.

Morris water maze task

The Morris water maze test was used to assess visual learning and memory. A dark-colored tank halfway filled with regular tap water. To experience the pool environment, rats were trained without platform sessions. Subsequently, the platform was hidden about 1 cm below the surface of water. The rats were placed in the maze from four equal quadrants (north, south, east, or west location). Rats were allowed to swim for 60 seconds to search for the escape platform. The trial was terminated when the platform was found. Rats were guided to stay on the platform for 15 s when they did not find the platform during the trial. The time to reach the platform (latency) and path lengths were recorded by software. Escape latency was recorded with a stopwatch. Platform crossing, target quadrant time, and target quadrant distance were also measured and recorded.

Hematoxylin and Eosin (HE) staining

The tissues were fixed with 4% paraformaldehyde and cut into 5 μm sections. Sections were deparaffinized and rehydrated and then stained with hematoxylin and eosin. The six fields of view were observed from each sample, and photographs were acquired under a microscope (Leica DMI6000B, Germany).

S-100β and NSE determinations

Blood samples were collected and refrigerated immediately and processed within 24 hours. Samples were centrifuged at 500 rpm and supernatants were stored at −80°C. S-100 calcium-binding protein B (S-100β) and neuron-specific enolase (NSE) were detected using enzyme-linked immunosorbent assay (ELISA) kits (Runyu, Shanghai, China).

Cytokine assays

Plasma serum was collected from the rats. Cytokine levels were examined using the following ELISA kits (Beyotime, Shanghai, China): Rat interleukin-6 (IL-6) ELISA Kit, Rat interleukin-10 (IL-10) ELISA Kit, Rat tumor necrosis factor-alpha (TNF-α) ELISA Kit and Rat interleukin-1beta (IL-1β) ELISA Kit. All samples were detected three times and average values were used in the analysis.

TdT-Mediated Nick-End Labeling (TUNEL)

The tissues were fixed with 4% formaldehyde. The tissues were then cut into 5-μm sections and the sections were incubated with TUNEL reagent (Beyotime, Shanghai, China). The nuclei were vstained using DAPI solution. Finally, the signals were measured with a florescent microscope (IX71, Olympus, Japan).

Western blot

Proteins from 6 rats were extracted using RIPA lysis buffer (Sangon, Shanghai, China) in each group. Then, about 15 micrograms protein extracts were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% skim milk at 37°C for 45 min and then covered with antibodies against ×(Bax) associated with Bcl-2 Bcl2 (Bax) (1:1000), B-cell lymphoma 2 (Bcl-2) (1:1000), p65 (1:2000), phosphorylated-p65 (p-p65) (1:1000), IkappaBalpha (IκBα) (1:1000) and β-actin (1:3000) (all from Abcam, Cambridge, MA, USA) at 4°C overnight. Secondary antibody (1:2000, Beyotime, Shanghai, China) was used to incubate the membranes at 37°C for 45 min. Immunoreactive proteins were determined by enhanced chemiluminescence (Beyotime, Shanghai, China), and protein bands were analyzed using Image J. β-actin was used as loading controls.

Statistical analysis

Data were expressed as means ± standard deviations (S.D.). GraphPad Prism 7.0 software was used to perform statistical analysis. Differences were evaluated using one-way ANOVA followed by Tukey. $p<0.05$ was considered statistically significant.

RESULTS

Cucurbitacin E improved brain injury in sevoflurane-induced rats

To detect the effects of cucurbitacin E on brain injury in sevoflurane-induced rats, brain tissues were collected. HE staining demonstrated that sevoflurane resulted in nuclear condensation and heteromorphism, and on average had a cytoplasmic reduction. However, treatment

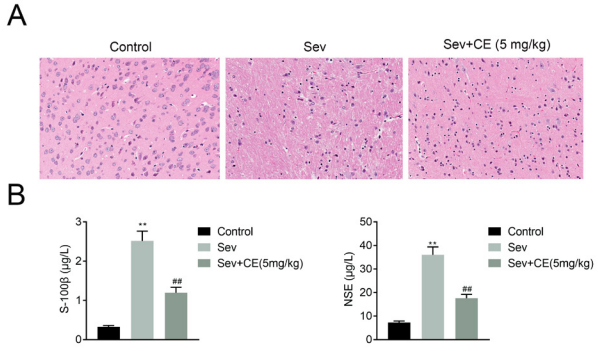

Figure 1. Cucurbitacin E improved brain injury in sevoflurane-induced rats
(**A**) HE staining examined pathological change of brain tissues. (**B**) S-100β and NSE levels were detected using ELISA assay. n=6. Note, $^{**}p<0.01$. $^{\#\#}p<0.01$. * *vs.* Control. # *vs.* Sev.

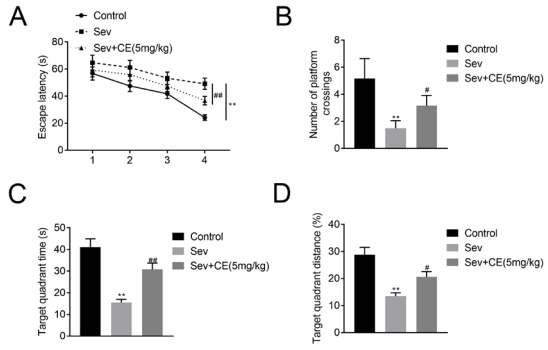

Figure 2. Cucurbitacin E attenuated cognition impairment in sevoflurane-induced rats
The Morris water maze task was performed to examine escape latency (**A**), platform crossing (**B**), target quadrant time (**C**), and target quadrant distance (**D**). n=6. Note, ***p*<0.01. ##*p*<0.01. * *vs.* Control. # *vs.* Sev.

with cucurbitacin E alleviated the pathological change of brain tissues induced by sevoflurane. Furthermore, normal neurons were still observed in the Sev + CE group (Fig. 1A). The ELISA assay showed that S-100β and NSE levels increased in the Sev group, while these decreased in the Sev + CE group (Fig. 1B). The data suggested that cucurbitacin E improved the brain injury induced by sevoflurane in rats.

Cucurbitacin E attenuated cognition impairment in sevoflurane-induced rats

To explore whether cucurbitacin E could function in cognition impairment in sevoflurane-induced rats, the morris water maze task was conducted. The results revealed that sevoflurane elevated escape latency (Fig. 2A), and reduced platform crossing (Fig. 2B), target quadrant time (Fig. 2C) and target quadrant distance (Fig. 2D). However, cucurbitacin E exhibited an opposite role in these effects induced by sevoflurane (Fig. 2A–D). These findings indicated that cucurbitacin E attenuated cognition impairment in rats induced by sevoflurane.

Cucurbitacin E alleviated sevoflurane-induced inflammation in rats

The effects of cucurbitacin E on sevoflurane-induced inflammation were further investigated and cytokine assays were carried out. ELISA assays verified that sevoflurane increased levels of IL-6, TNF-αand IL-1β (Fig. 3)

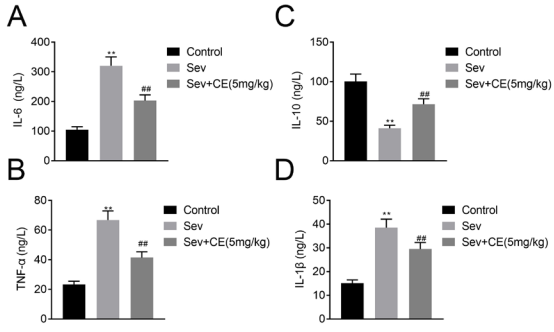

Figure 3. Cucurbitacin E alleviated sevoflurane-induced inflammation in rats
IL-6, IL-10, TNF-α and IL-1β level was examined using the ELISA kit. n=6. Note, ***p*<0.01. ##*p*<0.01. * *vs.* Control. # *vs.* Sev.

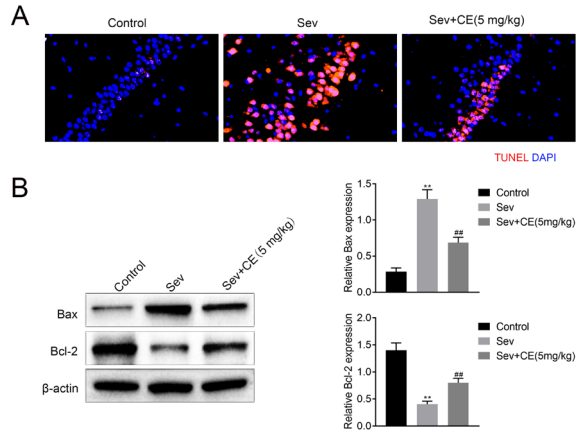

Figure 4. Cucurbitacin E downregulated sevoflurane-induced neuron apoptosis in rats
(**A**) TUNEL staining was used to detect apoptosis. (**B**) Western blot measured the levels of the Bax and Bcl-2 proteins. n=6. Note, ***p*<0.01. ##*p*<0.01. * *vs.* Control. # *vs.* Sev.

levels, and IL-1 (Fig. 3), and decreased the level of IL-10 (Fig. 3). However, cucurbitacin E downregulated the levels of IL-6, TNF-α and IL-1β levels, and upregulated the level of IL-10, which were induced by sevoflurane (Fig. 3). These results implied that cucurbitacin E alleviated sevoflurane-induced inflammation in rats.

Cucurbitacin E downregulated sevoflurane-induced neuron apoptosis in rats

We then examined how cucurbitacin E could affect sevoflurane-induced neuron apoptosis in rats. TUNEL staining proved that sevoflurane promoted neuron apoptosis, while sevoflurane-induced neuron apoptosis was alleviated in rats exposed to cucurbitacin E (Fig. 4A). Furthermore, Western blot showed that sevoflurane increased the level of Bax protein and decreased the level of Bcl-2 protein. However, cucurbitacin E treatment exerted an opposite role in these sevoflurane-induced protein levels (Fig. 4B), which were the same as the above results of the TUNEL staining experiment. Data indicated that cucurbitacin E downregulated sevoflurane-induced neuron apoptosis in rats.

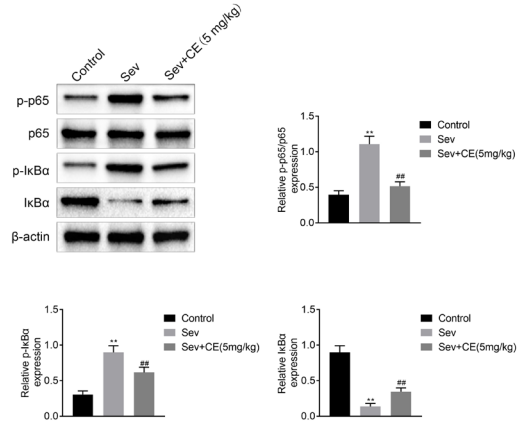

Figure 5. Cucurbitacin E regulated NF-κB pathway in sevoflurane-induced rats
The protein levels of p-p65, p65, p-IκBα and IκBα protein levels were examined using Western blot. n=6. Note, ***p*<0.01. ##*p*<0.01. * *vs.* Control. # *vs.* Sev.

Cucurbitacin E regulated NF-κB pathway in sevoflurane-induced rats

To measure the effects of cucurbitacin E on NF-κB pathway in sevoflurane-induced rats, Western blot was performed. The results demonstrated that sevoflurane enhanced p-p65/p65 and p-IκBα protein levels, as well as reduced IκBα protein level. However, these sevoflurane-induced protein levels were reversed when rats were treated with cucurbitacin E (Fig. 5). These findings suggested that cucurbitacin E regulated NF-κB pathway in sevoflurane-induced rats.

DISCUSSION

In this study, cucurbitacin E improved brain injury in brain tissues of sevoflurane-induced rats. We found that cucurbitacin E treatment attenuated sevoflurane-induced cognition impairment in rats. Sevoflurane promoted inflammation in rats, whereas cucurbitacin E showed opposite effects. Sevoflurane-induced neuron apoptosis could be reduced with cucurbitacin E. Further, NF-κB pathway was inhibited in sevoflurane-induced rats exposed to cucurbitacin E. These findings indicated that cucurbitacin E exhibited an important role in sevoflurane-induced cognitive function in rats.

Previously, sevoflurane was found to cause cognitive dysfunction by activating inflammation and cell death in rats (Cui *et al.*, 2018). Furthermore, sevoflurane had induced apoptosis, increased ER stress, and cognitive dysfunction (Chen *et al.,* 2013). In our study, sevoflurane probably elevated brain injury and led to cognitive dysfunction. Additionally, sevoflurane increased inflammation and inhibited neuron apoptosis in rats. Cucurbitacin E has been reported to be involved in inhibition of cell invasion and migration (Zhang *et al.*, 2020). Moreover, cucurbitacin E also exhibited anti-inflammatory and anti-proliferative properties, which were mediated by its role in the polymerization of the actin cytoskeleton (Momma *et al.*, 2008; Sorensen *et al.*, 2012). Interestingly, we demonstrated that cucurbitacin E alleviated sevoflurane-induced inflammation, accompanied by down-regulation of IL-6, TNF-α and IL-1β, and up-regulation of IL-10. Cucurbitacin E also attenuated neuronal apoptosis in sevoflurane-induced rats, evidenced by increased Bax protein level and decreased Bcl-2 protein level. Additionally, cucurbitacin E has been reported to have neuroprotective properties in a model of Parkinson's disease through regulation of autophagy (Arel-Dubeau *et al.*, 2014). Consequently, our study revealed that cucurbitacin E improved brain injury and attenuated cognition impairment, including escape latency inhibition, increased platform crossing, elevation of target quadrant time, upregulation of target quadrant distance. These results showed that cucurbitacin E improved sevoflurane-induced cognitive function in rats.

Accumulating evidence verified that astrogliosis promoted the pro-inflammatory NF-kB pathway, while transgenic inhibition of NF-kB signaling could protect against neurodegeneration and cognitive impairment in reactive astrocytes (Saggu *et al.*, 2016). NF-κB protein, as the core inflammatory molecule, was widely expressed in mammals and participated in the start or regulation of inflammation. Additionally, NF-κB protein has been found to regulate inflammatory factors and promote inflammatory reactions (Mitchell & Carmody, 2018). NF-κB signaling activation was featured by up-regulating the phosphorylation of IκBα and NF-κB p65 expression. Activated NF-κB could be involved in increasing immune reactions, inflammatory reactions, cell apoptosis, and aging (Meffert & Baltimore, 2005; Vaughan & Jat, 2011). Furthermore, phosphorylation and degradation of IκBα could suppress subsequent nuclear translocation of NF-κB p65. Importantly, in our study, the results showed that sevoflurane promoted NF-κB activation, while cucurbitacin E exerted opposite effects on NF-κB activation. The data suggested that cucurbitacin E negatively regulated the cognitive dysfunction induced by sevoflurane in rats by regulating NF-κB pathway.

However, the molecular mechanism of cucurbitacin E in sevoflurane-induced PND needs to be further investigated. Moreover, we do not explore how cucurbitacin E is applied to the clinical sample. Therefore, additional studies will be conducted in the future.

In conclusion, we verified that cucurbitacin E improved brain injury and cognitive impairment and reduced inflammation and neuronal apoptosis in rats induced by sevoflurane through NF-κB pathway. This study provides a new basis for the beneficial effects of cucurbitacin E administration during PND treatment.

Declarations

Acknowledgements. Not applicable.
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Competing interests. The authors state that there are no conflicts of interest to disclose.
Ethics approval. Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University.
Contribution of authors. Shaohua Zheng and Bowen Shi designed the study, supervised the data collection, Xin Li analyzed the data, interpreted the data, Hui Yuan, Yinglu Feng prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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