

Original Research Article

Carnosol ameliorates sevoflurane-induced cognitive impairment in aged rats by suppressing NLRP3 inflammasome via activation of autophagy

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Abstract

Purpose: To investigate the effect of carnosol on the progression of postoperative cognitive dysfunction (POCD) and to elucidate its mechanism of action.

Methods: A cognitive impairment model rat induced by sevoflurane (SEV) was established. Dihydroethidium (DHE) assay was conducted to determine the effect of carnosol on reactive oxygen species (ROS) levels in sevoflurane-induced rats. Adenosine triphosphate (ATP) production and immunoblot assays were used to assess carnosol effect on mitochondrial damage, while water maze experiment was performed to evaluate cognitive dysfunction in the rats. The mechanism of action was investigated using immunoblot assay.

Results: Carnosol suppressed sevoflurane-induced ROS production in the rats, and alleviated sevoflurane-induced mitochondrial damage; it also activated autophagy. Furthermore, carnosol suppressed SEV-induced NLRP3 inflammasome activation by activating autophagy and suppressing SEV-induced cognitive dysfunction via the suppression of NLRP3 inflammasome activation.

Conclusion: Carnosol ameliorates SEV-stimulated cognitive impairment by suppressing NLRP3 inflammasome and thus, can potentially be developed serve as a therapeutic agent for the management of cognitive impairment.

Keywords: Carnosol, Postoperative cognitive dysfunction (POCD), Sevoflurane (SEV), NLRP3 inflammasome, Autophagy

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INTRODUCTION

Postoperative cognitive dysfunction (POCD) is a type of complication with long-term consequences, defined as impaired memory, attention and information processing that occurs

after anesthesia [1]. Studies show that about 10 % of surgery patients as well as 40 % of older patients over the age of 65 develop POCD [2]. Compared to patients without POCD, patients with POCD have significantly higher mortality rates and higher dependence on social security

[3]. Despite extensive research efforts, the pathogenesis of POCD remains unknown [4]. New therapeutic agents still need to be developed to treat POCD.

Rosemary (*Salvia rosmarinus*) is a Mediterranean plant that is now widely available in several countries [5,6]. Rosemary extract can be used to treat inflammation-related diseases [7]. Carnosol is a phenolic diterpenoid which exists in rosemary extract and has been shown to have anti-inflammatory and antioxidant effects [8]. Carnosol treatment inhibited the eosinophils in the bronchoalveolar lavage fluid of mice after ovalbumin treatment [9]. Previous studies have shown that carnosol alleviates sevoflurane-stimulated cognitive dysfunction in aged rats via NF- κ B pathway. It also causes the activation of autophagy in breast cancer [8]. However, the potential effect and mechanism of action of the compound on POCD remains unknown.

There is growing evidence of a causal relationship between SEV-stimulated cognitive impairment and NLRP3 inflammasome [10]. In response to cellular stress, NLRP3 recruits ASC as well as pro-caspase-1, which causes the cleaved caspase-1 to activate and process the maturation of IL-1 β and IL-18 [11]. NF- κ B is involved in the control of many cellular processes [11]. The activation of NF- κ B family is a key step in regulating pyroptosis. However, the effects of carnosol on autophagy and its relationship with NLRP3 is unclear. The aim of this study was to investigate the role of carnosol in POCD progression using a sevoflurane-induced cognitive impairment rat model of rats.

EXPERIMENTAL

POCD rat model

Male Sprague–Dawley rats (12 months old, 250 – 280 g) were purchased from Vital River (Beijing, China). The procedures in this study were approved by the Ethics Committee of Xi'an International Medical Center Hospital, Shanxi, China (approval no. 202315), and conducted in accordance with the guidelines of Declaration of Helsinki in Biomedical and Behavioral Research [12].

For the establishment of POCD model, sevoflurane (2 % SEV) was inhaled by old rats (8 months old) for 5 h, and carnosol administered to the rats in doses of 5 and 10 mg/kg every 2 days, respectively. The rats were divided into the following groups: control, SEV, SEV + carnosol (5 mg/kg), as well as SEV + carnosol (10 mg/kg) groups.

Morris water maze (MWM) tests

The MWM tests were performed in a swimming pool with four quadrants and the activity of the rats was recorded. The rats were trained for 4 days in order to determine the location of the platform; if the rats could not find the location within 90 s, they were placed on the platform. Finally, the platform was removed. The time and number was recorded.

Determination of ROS

For ROS staining, the cells were mounted and stained with Dihydroethidium (DHE). DHE fluorescence of 20 cells per field in five random fields was quantified by the use of AxioVision software.

JC-1 staining

To analyze mitochondria damage, samples were placed in 12-well plates and kept for 24 hours for cell adhesion. After rinsing, the cells were incubated with 2 μ M JC-1 for 15 min at 37 °C. After rinsing in phosphate buffer (PBS) 3 times, the cells were photographed and the JC-1 positive cells were counted per field.

Determination of ATP production

For the determination of ATP production, an ATP detection kit (Beyotime, Beijing, China) was utilized in accordance with the manufacturer's protocol.

Immunoblot assay

The samples were electrophoresed and transferred onto PVDF membranes and then blocked with 5 % fat-free milk. Subsequently, the membranes were conjugated with antibodies targeting LC3 (ab192890, 1:1000, Abcam), p62 (ab109012, 1:1000, Abcam), NLRP3 (ab263899, 1:1000, Abcam), ASC (ab283684, 1:500, Abcam), Caspase-1 (ab207802, 1:500, Abcam), IL-18 (ab243091, 1:1000, Abcam), IL-1 β (ab254360, 1:1000, Abcam), and β -actin (ab8226, 1:3000, Abcam) for 2 h at room temperature. Subsequently the membranes were incubated with secondary antibodies (1:2000, Abcam) for 1 h at room temperature. The blots were then analyzed with ECL kit.

Statistics

Statistical analysis was performed with GraphPad 7.0 software using t-test, and the data

are shown as mean \pm SD. $P < 0.05$ was considered statistically significant.

RESULTS

Carnosol suppressed sevoflurane-induced ROS production of rats

The results showed that carnosol treatment decreased DHE expression levels in sevoflurane-induced hippocampal neurons, suggesting the inhibition of ROS production (Figure 1). Therefore, Carnosol suppressed sevoflurane-induced ROS production of rats.

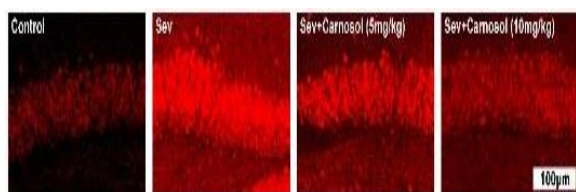


Figure 1: Carnosol suppressed sevoflurane induced ROS production of rats. DHE was used to detect ROS levels in hippocampus from control, SEV, SEV+carnosol (5 mg/kg), and SEV+carnosol (10 mg/kg) groups. Scale bar indicates 100 μ m

Carnosol suppressed mitochondrial damage induced by sevoflurane, and activated autophagy

Immunostaining showed the degree of mitochondrial damage of the hippocampus from each group. The aggregates and monomers reflected the degree of mitochondrial damage. The data showed that the monomers were enhanced and the aggregates decreased in SEV hippocampal neurons (Figure 2 A). In addition, carnosol reduced the monomers and increased the aggregates in SEV-induced hippocampal neurons, suggesting the inhibition of mitochondrial damage (Figure 2 A). Similarly, carnosol decreased ATP production in SEV hippocampal neurons but induced the production of ATP in SEV hippocampal neurons (Figure 2 B). Western blot results showed that the ratio of LC3BII/LC3BI in SEV hippocampal neurons increased, while carnosol decreased the ratio in SEV hippocampal neurons, suggesting the inhibition of autophagy (Figure 2 C). Interestingly, increased expression of p62 was found in SEV hippocampal neurons but carnosol decreased p62 expression in SEV hippocampal neurons, further confirming the previous data (Figure 2 D). Therefore, carnosol alleviated mitochondrial damage induced by sevoflurane and activated autophagy.

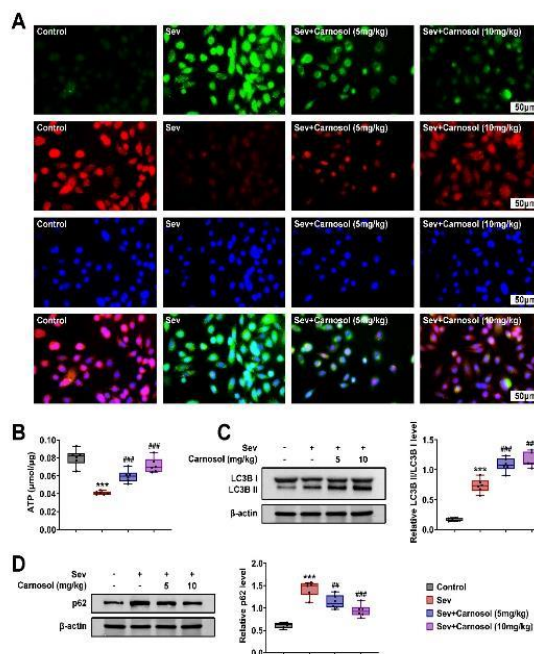


Figure 2: Carnosol suppressed mitochondrial damage induced by sevoflurane and activates autophagy. (A). Immunostaining showed the degree of mitochondrial damage of hippocampus from the indicated groups. Green indicates monomers; Red indicates aggregates. Scale bar indicates 50 μ m; (B). ATP production was shown in hippocampus from the indicated groups; (C). Immunoblot assays showed the expression levels of LC3B in hippocampus from the indicated groups. The ratio of LC3BII/LC3BI was calculated; (D). Immunoblot assays showed the expression levels of p62 in hippocampus from the indicated groups. Data are presented as mean \pm SD; *** $P < 0.001$, SEV vs control, ## $p < 0.01$, ### $p < 0.001$, SEV+carnosol vs SEV

Carnosol suppressed sevoflurane-induced NLRP3 inflammasome activation

To determine the potential mechanism of carnosol-mediated effect in sevoflurane induced rats, the role of carnosol in the NLRP3 pathway was assessed after treatment with SEV, carnosol and the inhibitor of autophagy, 3-MA. Carnosol increased the levels of NLRP3 in SEV group was found, and reversed the increase of NLRP3 expression caused by SEV treatment (Figure 3 A). However, 3-MA treatment further increased the expression of NLRP3 in carnosol-treated rats, suggesting the regulation of autophagy in NLRP3 pathway (Figure 3 A). Furthermore, NLRP3 signaling pathway was activated following the establishment of SEV-induced model as demonstrated by increased levels of ASC, IL-1 β and IL-18 (Figure 3 B). The decreased levels of ASC, IL-1 β and IL-18 levels were caused by carnosol treatment (Figure 3 B). 3-MA treatment increased further the expression of NLRP3 in carnosol-treated rats. Thus, the data

suggest that carnosol suppressed sevoflurane-induced NLRP3 inflammasome activation by regulating autophagy.

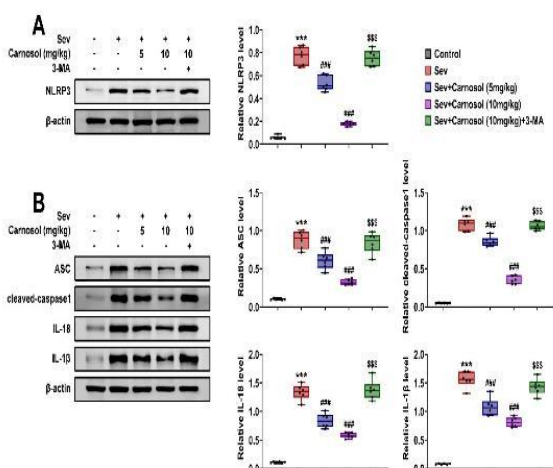


Figure 3: Carnosol suppressed sevoflurane induced NLRP3 inflammasome activation by activating autophagy. (A). Immunoblot assays showed the expression levels of NLRP3 in hippocampus from the indicated groups; (B). Immunoblot assays showed the expression levels of indicated proteins in the hippocampus from the indicated groups. Data are presented as mean ± SD, *** $P < 0.001$, SEV vs control, ### $p < 0.001$, SEV+carnosol vs SEV, \$\$\$ $p < 0.001$, SEV+carnosol+3-MA vs SEV+carnosol

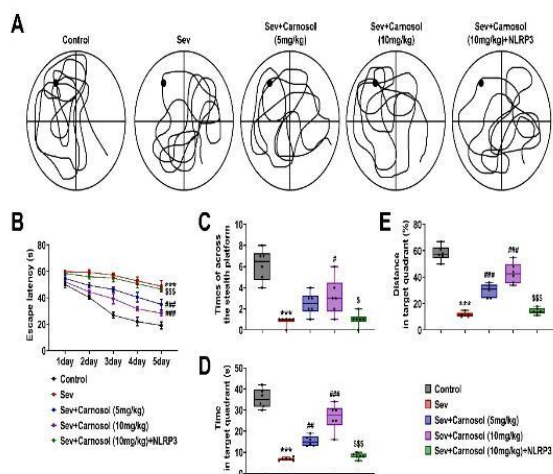


Figure 4: Carnosol suppressed sevoflurane induced cognitive dysfunction by inhibiting NLRP3 inflammasome activation. (A). Water maze experiment reveals rat movement track from the indicated groups; (B-E). Escape latency (B), time of across the stealth platform (C), time in target quadrant (D), and distance in target quadrant (E) of rats from the indicated groups. Data are presented as mean ± SD; *** $p < 0.001$, SEV vs control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, SEV+carnosol vs SEV, \$ $p < 0.05$, \$\$\$ $p < 0.001$, SEV+carnosol+3-MA vs SEV+carnosol

Carnosol suppresses sevoflurane-induced cognitive dysfunction by inhibiting NLRP3 inflammasome activation

As expected, the cognitive ability of rats in SEV group was impaired significantly with poor ability to escape latency, locate the target, long latency time and longer time in the target quadrant (Figure 4 A - E). Carnosol treatment improved the performance of rats (Figure 4 B - E). Interestingly, NLRP3 treatment further impaired the performance of rats, suggesting that carnosol alleviated cognitive dysfunction (Figure 4 B - E). Therefore, carnosol suppressed SEV-induced cognitive dysfunction by suppressing NLRP3 inflammasome activation.

DISCUSSION

With the different types of surgery and the duration of general anesthesia, the degree of POCD varies. In recent years, there has been great progress in anesthesia technology, monitoring instruments and surgical operations, and these have greatly improved the safety of surgery, POCD is still common and its pathogenesis and mechanisms have attracted wide attention [10]. POCD can usually be treated with medication [10]. If the patient has cognitive dysfunction after surgery, it can be treated with drugs, as the main purpose is to improve the patient's condition and facilitate recovery [10]. With the aid of intravenous drip, nutritional neurodrugs and other treatments to ameliorate brain tissue ischemia and hypoxia, and postoperative recovery, using mecobalamine tablets, adenosine monophosphate and other drugs, may improve the cognitive ability of patients [13]. However, it is still necessary to find more effective drugs. Here, carnosol was shown to ameliorate the sevoflurane-induced cognitive impairment in aged rats. Therefore, carnosol may serve as a drug for the management of POCD.

Carnosol is a diterpenoid phenolic compound, which has antioxidant, anti-inflammatory, anti-proliferation and anti-tumor effects [14,15]. Due to its remarkable pharmacological activity, more and more scholars at home and abroad have paid more attention to it in recent years [8]. It has a potential role in the prevention and treatment of neuropathic diseases [8]. Carnosol is a potent ribosome S6 kinase (RSK2) inhibitor and also an carnosol is also an Nrf2 activator, increasing Nrf2 levels and promoting heme oxygenase-1 (HMOX1) expression [16]. It alleviated mitochondrial damage induced by SEV and also activated autophagy and increased SEV-induced NLRP3 inflammasome activation by activating autophagy. Carnosol ameliorated sevoflurane-

induced cognitive impairment. Previous studies have shown that it alleviated sevoflurane-induced cognitive dysfunction in aged rats by mediating NF- κ B pathway [16]. In the present study, carnosol alleviated sevoflurane-induced cognitive dysfunction by regulating NLRP3 inflammasome as well as autophagy. However, further *in vivo* studies to elucidate the mechanism of action are still needed. Autophagy is vital in the elimination of protein aggregates and abnormal organelles [10]. Enhanced autophagy disrupts neuronal homeostasis [17]. Activation of autophagy suppresses the formation of NLRP3 inflammasome [10]. NLRP3 inflammasome is highly expressed and over-activated in aged rats, and is vital in the central inflammatory response [18]. Over-activation of NLRP3 inflammasome leads to a variety of diseases, including POCD [10]. NLRP3 inflammasomes are involved in the important mechanisms of POCD [10]. It also reduced cognitive impairment in older rats. Interestingly, carnosol further improved SEV-induced NLRP3 inflammasome activation by activating autophagy, thus ameliorating POCD. However, the precise mechanism of action needs to be investigated further.

CONCLUSION

Carnosol ameliorates SEV-induced cognitive impairment in aged rats by suppressing NLRP3 inflammasome, as well as autophagy. Therefore, carnosol is a promising drug for the management of POCD.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Simin Zheng and Hongfei Xiong designed the study and carried them out, Simin Zheng, Siyuan Li, Hongtao Liu, Yunpeng Teng and Ying Liu supervised the data collection, analyzed and interpreted the data; Simin Zheng and Hongfei Xiong prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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