Iranian Journal of Basic Medical Sciences
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Rosemary inhalation improves the behavior and cognition of vascular cognitive impairment rats by regulating SIRT1/mTOR pathway-mediated cell apoptosis and autophagy

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ARTICLE INFO

Article type:
Original

Article history:
Received: Sep 26, 2025
Accepted: May 23, 2026

Keywords:
Rosemary oil
Cognitive impairment
SIRT1
Apoptosis
Autophagy

ABSTRACT

Objective(s): Vascular cognitive impairment (VCI) is a major cause of behavioral and cognitive abnormalities and dementia, but there are currently no specific drugs for VCI. Rosemary oil has been found to have a role in cognitive function. This study aims to evaluate whether inhalation of rosemary can improve learning and memory in VCI rats by regulating neuronal apoptosis and autophagy.

Materials and Methods: Male SD rats were used to establish a VCI rat model by bilateral common carotid artery ligation. The rats were intervened with rosemary essential oil inhalation and EX527 injection. Cognitive function was evaluated by the Morris water maze test two weeks after the operation. The pathological changes of hippocampal tissue (HE, Nissl, TEM, TUNEL) and protein expression (WB) were analyzed.

Results: Compared with the sham group, the VCI group exhibited cognitive deficits, neuronal loss, mitochondrial damage, and increased neuronal apoptosis ($P<0.001$). The levels of caspase-3, LC3B, and p-mTOR/mTOR in the hippocampal tissue were significantly elevated ($P<0.0001$, $P<0.01$, $P<0.01$), while the protein level of SIRT1 was significantly reduced ($P<0.01$). Compared with the VCI group, the rosemary group showed improved cognitive ability, ameliorated neuronal damage, and reduced neuronal apoptosis ($P<0.05$). The levels of caspase-3, LC3B, and p-mTOR/mTOR were decreased ($P<0.0001$, $P<0.01$, $P<0.05$), while the level of SIRT1 was increased ($P<0.05$). However, intervention with EX527 attenuated this protective effect.

Conclusion: Rosemary inhalation can improve behavior and cognition in VCI rats by regulating SIRT1/mTOR pathway-mediated cell apoptosis and autophagy.

▶Please cite this article as:
Shi F, Hong E, Bao J, Ye W, Wang L, Wang H, Zhang Y. Rosemary inhalation improves the behavior and cognition of vascular cognitive impairment rats by regulating SIRT1/mTOR pathway-mediated cell apoptosis and autophagy. Iran J Basic Med Sci 2026; 29:

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Introduction

Vascular cognitive impairment (VCI), as a recently recognized disease, covers a range of cognitive disorders from mild cognitive impairment to vascular dementia (1). It is considered the second leading cause of dementia, affecting 20-40% of dementia patients (2). The incidence rate of VCI increases year by year, and VCI has become one of the major public health challenges in the world. According to 2018 statistics, the number of people affected by VCI has reached 50 million, and it is projected to triple by 2050 (3). VCI is mainly caused by cerebrovascular diseases (4, 5). Age and vascular risk factors can increase the probability of VCI (6). It is shown that the incidence rate of VCI in late age can be predicted by vascular risk factors in midlife. Hypertension, high cholesterol, diabetes, and smoking will increase the probability of VCI by 20% to 40%, and each vascular risk factor will overlap (6). In late life, Alzheimer's disease (AD) and other neurodegenerative diseases increase the probability of VCI occurrence, and VCI can intertwine with these diseases, exacerbating cognitive impairment (7). At present, there is no specific treatment method for VCI. The treatment of VCI mostly adopts drug therapy that solves cognitive and behavioral symptoms, combined with non-drug therapy that optimizes the quality of life (8). Prevention of VCI is more important than treatment. Reducing vascular risk factors and maintaining healthy lifestyle habits are considered the most promising approaches to preventing and treating VCI (9). Given the current lack of methods for treating VCI, in-depth research into its underlying mechanisms and the search for drugs to treat it will help us better understand and develop new methods for VCI treatment, and such research is of great significance.

Rosemary, as an ancient folk herb, acts on various conditions, including headache, stomach pain, neurogenic agitation, and memory improvement (10). Therefore, rosemary is also known as a memory herb and enjoys a special position in folk medicine. Numerous studies have shown that rosemary has antibacterial, anti-inflammatory, anti-oxidant, and cognitive improvement effects in both animals and cells (11-13). It has been shown that incubating

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SH-SY5Y neuroblasts with 10 μ M rosemary can increase the expression of nuclear factor-E2-related factor 2 (Nrf2) in cells, thereby inhibiting cell damage caused by oxidative stress (14). Rosemary has a cognitive-improving effect. It has been shown that feeding middle-aged rats rosemary extract can increase their spatial memory scores, and that the activities of superoxide dismutase and glutathione peroxidase 4 in the hippocampus were increased, indicating that rosemary can improve behavioral cognition in middle-aged rats by inhibiting iron death and oxidative stress (15). Rosemary has a good therapeutic effect on neurological diseases. It is reported that rosemary has a therapeutic effect on AD by preventing amyloid beta aggregation, and it has the potential to be used as a drug for treating AD and vascular dementia (16). Essential oils are plant-derived mixtures characterized by volatility. Aromatherapy with essential oils can treat various diseases, such as insomnia and headaches. A study showed that the use of rosemary essential oil can improve sleep quality and sleep disorders (17). Other studies have shown that inhalation of rosemary can improve learning and cognitive abilities in VCI rats by increasing levels of 5-hydroxytryptamine and gamma-aminobutyric acid in the hippocampus (18). Although rosemary has therapeutic effects on nervous system diseases, and the inhalation of rosemary essential oil can improve sleep, whether rosemary essential oil has therapeutic effects on VCI is unknown. In this study, we explored the effect of rosemary essential oil on VCI and its underlying mechanism, laying the foundation for the development of new treatment methods for VCI.

Materials and Methods

Experimental animals

6-8-week-old male SD rats were purchased from Sibeifu (Beijing) Biotechnology Co., Ltd., with license number SCXK (Beijing) 2024-0001.

Experimental reagents

Rosemary essential oil (Zhejiang); silent mating type information regulation 1 (SIRT1) inhibitor EX527 (selleck; S1541); Xylene (33535, Xilong Science Co., Ltd.); Hematoxylin bluing solution (G1040, Solarbio); Anhydrous ethanol (3206, Xilong Science Co., Ltd.); 95% ethanol (32061, Xilong Science Co., Ltd.); Eosin staining solution (G1100, Soleibal); Hematoxylin staining solution (ZLI-9610, Beijing Zhong Shan -Golden Bridge Biological Technology CO.,LTD); Ultra clean advanced sealing adhesive (YZB, BASO); TUNEL detection kit (red) (C1090, Beyotime Biotechnology); Nissl staining reagent (G1036-100mL, SERVICEBIO); Sodium citrate, dihydrate (S116311, Aladdin); Citric acid (77-92-9, Tianjin Damao Chemical Reagent Factory); Bovine serum albumin (BSA) (S12012, Shanghai yuanye Bio-Technology Co., Ltd); Ready to use DAPI staining solution (KGA215-50, Jiangsu KeyGEN BioTECH Corp., Ltd); Rabbit anti-LC3B antibody (Affinity,AF4650);Secondary antibody: Cy3 Goat Anti-Rabbit IgG (H+L) (AS007, ABclonal); RIPA cell lysate (C1053, Beijing Pulilai Gene Technology Co., Ltd.); Ultra-sensitive chromogenic solution (RJ239676, Thermo Fisher Scientific); Internal reference antibody: Mouse anti -β- actin (HC201, TransGen Biotech); Secondary antibody: Goat Anti-Mouse IgG (H+L) (GB23301, Servicebio); Primary antibodies: Rabbit Anti caspase-3(YM8058, Immunoway); Rabbit Anti SIRT1(ab110304, abcam); Rabbit Anti LC3B (A19665, ABclonal); Mouse Anti mammalian target protein of rapamycin (mTOR) (66888-1-Ig, Proteintech); Rabbit Anti p-mTOR (YM8326, Immunoway); Secondary antibody: Goat Anti Rabbit IgG (H+L) (GB23303, Servicebio).

Experimental instrument

Embedding machine (HistoCore Arcadia, Leica); Slicer (HistoCore BIOCUT, Leica); Spreader (Leica HI1210, Leica); Pressure cooker (YS20ED, Supor); Dehydrator (KD-TS3S1, Kedi Instrument Equipment Co., Ltd.); Centrifuge (5424R, Eppendorf); Shaker (HY-2B, Changzhou Langyue Instrument Manufacturing Co., Ltd.); Microscope (CX43, Olympus); Transmission electron microscope (JEM-1400, Japan Electron); Fluorescence microscope (BX53, Olympus); Microplate reader (SuPerMax 3100, Shanghai Sanpu); Chemiluminescence Image Analysis System (Tanon-5200, Shanghai Tianneng Technology Co., Ltd.)

Animal model construction

Six to eight week-old male SD rats were housed in an SPF environment at 20-26 °C and allowed to eat and drink freely. After one week of adaptive feeding, the rat model of VCI was constructed. Following anesthesia with intraperitoneal injection of sodium pentobarbital (50 mg/kg), the vertebral arteries on both sides were scalded, the common carotid arteries on both sides were ligated for 10 min, loosened for 5 min, and then ligated again for 3 cycles(19). The rats in the sham group (n=6) only underwent carotid artery dissection. After the surgery, the wound was sutured, and 0.1 mL of penicillin (200000 U/mL) was injected to prevent wound infection. After modeling, the rats were randomly divided into: 1) VCI group (n=6), 2) Rosemary group (n=6), and 3) Rosemary+SIRT1 inhibitor group (n=6). Rats in the Rosemary group were given rosemary inhalation. 5.5 mL of rosemary essential oil was added to the absolute ethanol to dissolve it to 550 mL. In a relatively sealed test box (48 cm × 35 cm × 20 cm), an incense burner was used to atomize 1 mL of 1% rosemary solution, and a thin sieve plate was laid on top. Place the rats on a thin sieve tray after the drug has fully evaporated and diffused throughout the entire test box, and cover the test box lid. 30 min later, the rats were removed and given rosemary for inhalation twice a day. Rats in the Rosemary+SIRT1 inhibitor group were treated with rosemary inhalation therapy, followed by a once-daily intraperitoneal injection of EX527 (50 μg/kg) (20) as an intervention. The intervention lasted for 14 days. Two weeks after surgery, the Morris water maze test was performed. The rats were then anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and euthanized by exsanguination. The whole brain was rapidly removed and rinsed in pre-cooled (4 °C) normal saline to clean the blood from the surface. After separation, the brain tissue was blotted dry with filter paper, placed into cryovials, and quickly frozen in liquid nitrogen. The samples were finally stored at -80 °C for subsequent use. The experimental procedure is illustrated in Figure 1 below.

Water maze test

After 2 weeks of treatment, the water maze test was conducted on SD rats. The black circular pool was filled with water (23–25 °C), and a platform was placed inside the pool. The experiment consisted of two phases and lasted for a total of 6 days. The first 5 days constituted the acquisition phase, during which the animals were placed into the

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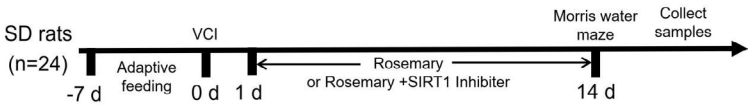

Figure 1. Timeline of the animal experimental procedure
VCI: Vascular cognitive impairment

water from different starting points each day, forcing them to locate the platform. If a rat failed to find the platform within 60 sec, the experimenter guided it to climb onto the platform. A video-tracking system was used to record the rats' movement trajectories, and platform-reach latency was extracted from the recorded data. The 6th day was the test phase, during which the platform was removed. The rats were allowed to explore the pool freely after being placed in the water, and the number of platform crossings was recorded using the video-tracking system (21, 22).

HE staining of rat brain tissues

The rat brain tissue was fixed and embedded in paraffin, and the embedded tissue was sliced. The slices were dried in a drying oven, deparaffinized in xylene and ethanol, stained with hematoxylin for 3-5 min, washed with water, and then differentiated with 1% hydrochloric acid alcohol. After washing with water, the anti-blue solution was added. The slices were washed with water, stained with eosin for 3-5 min, washed with water, and dehydrated through a series of ethanol concentrations. After sealing, the slices were observed and photographed under a microscope (CX43, Olympus).

Nissl staining of neurons in rat brain tissues

After baking, deparaffinization, and hydration, the rat brain tissue paraffin sections were stained with Nissl staining solution for 2–5 min and washed. The excess water was discarded, and the sections were dried in a 65 °C oven. After transparency with xylene, the sections were sealed with neutral resin, and images were observed and collected under a microscope.

Observation of mitochondrial structure in the hippocampus of rats by transmission electron microscopy

Rat hippocampal tissues were fixed with glutaraldehyde, then fixed again with citric acid, and dehydrated on ice. After acetone treatment, the tissue was embedded in epoxy resin and incubated at 37 °C overnight, 45 °C for 12 hr, and 60 °C for 48 hr. The embedded tissue was cut into 70 nm sections. Finally, double-staining was performed with 3% uranyl acetate and lead citrate, and the sections were observed and photographed under transmission electron microscopy (JEM-1230, JEOL, Japan).

TUNEL staining of cell apoptosis

After baking, dewaxing, and hydration, 20 μg/mL proteinase K solution was added dropwise to the rat brain tissue paraffin sections and incubated in a constant temperature incubator (37 °C). The TUNEL detection solution was added to the sections and incubated for 1 hr in a constant-temperature incubator (37 °C). The sections were washed with PBS, and DAPI was added dropwise to stain the nuclei for 5 min. The sections were sealed and observed under a fluorescence microscope.

Immunofluorescence detection

Paraffin sections of rat brain tissue were baked, dewaxed, and placed in a repair solution for antigen repair. After blocking with 5% BSA, the sections were incubated overnight at 4 °C with the LC3B antibody (Affinity, AF4650, 1/200). After washing, the fluorescent secondary antibody, Cy3 Goat Anti-Rabbit IgG (H+L), was added dropwise. The sections were then incubated at 37 °C for 45 min, counterstained with DAPI, sealed, and observed under a fluorescence microscope.

Western blot

RIPA lysis solution was added to rat hippocampal tissues, which were then ground and sonicated. After centrifugation for 10 min at 1200 r/min in a high-speed centrifuge at 4 °C, the supernatant was collected for total protein quantification using the BCA protein quantification kit according to the instructions. The total protein was added to the loading buffer and boiled for 10 min for denaturation, and then the sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out, followed by membrane transfer. The constant current was 300 mA. The membrane was blocked with 5% skim milk, incubated with the primary antibody overnight at 4 °C, and then incubated with the secondary antibody at room temperature for 2 hr the next day. The membrane was immersed in the luminous solution and then placed on the stage of the chemiluminescence imaging system for development and photography. The antibodies used in this study and the dilution ratio are shown in Table 1.

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Table 1. List of primary and secondary antibodies used in this study and their respective dilution ratios

Antibody	Dilution ratio
Mouse Anti-β-Actin（HC201，TransGen Biotech）	1:2000
Rabbit Anti Caspase-3（YM8058，Immunoway）	1:2000
Rabbit Anti SIRT1（ab110304，abcam）	1:2000
Rabbit Anti LC3B（A19665，ABclonal）	1:2000
Mouse Anti mTOR（66888-1-Ig，Proteintech）	1:20000
Rabbit Anti P-mTOR（YM8326，Immunoway）	1:5000
HRP conjugated Goat Anti-Mouse IgG (H+L)（GB23301，Servicebio）	1:2000
HRP conjugated Goat Anti-Rabbit IgG (H+L)（GB23303，Servicebio）	1:2000

SIRT1: Silent mating type information regulation 1; LC3B: Microtubule-associated protein 1 light chain 3; mTOR: Mammalian target protein of rapamycin

Statistical analysis

All experiments were independently repeated at least three times. Prior to statistical analysis, the normality of the data was assessed using the Shapiro–Wilk test, and homogeneity of variance was examined using Levene's test. After confirming that the data met the assumptions of normality and homogeneity of variance, results were expressed as mean ± standard deviation (± SD). Statistical analysis and graphing were performed using GraphPad Prism 8.0 software. Differences among multiple groups were analyzed using one-way analysis of variance, followed by Tukey's test for post hoc comparisons. The significance level was set at $\alpha = 0.05$, and $P<0.05$ indicates a significant difference.

Results

Rosemary improves the behavioral cognition of VCI rats

The behavioral cognition of rats was detected by the water maze test. As shown in Figure 2, compared with the sham group, the VCI group showed significantly prolonged escape latency ($P<0.05$) and significantly reduced number of platform crossings ($P<0.0001$). Compared with the VCI group, the rosemary group had shorter escape latency ($P<0.05$) and an increased number of platform crossings ($P<0.01$). Compared with the rosemary group, the group treated with rosemary and SIRT1 inhibitors had a longer escape latency ($P>0.05$) and a reduced number of platform crossings ($P>0.05$). The results indicated that rosemary has a therapeutic effect on the behavioral and cognitive functions of VCI rats.

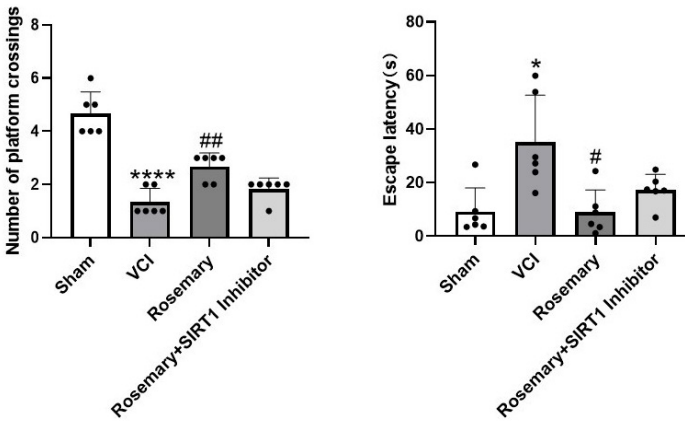

Figure 2. Behavioral cognition of rats was detected by the water maze test (*$P<0.05$, ****$P<0.0001$ compared with the Sham group; #$P<0.05$, ##$P<0.01$, compared with the Model group, n=6)
SIRT1: Silent mating type information regulation 1; VCI: Vascular cognitive impairment

Effects of Rosemary on neurons in the brain tissues of VCI rats

HE and Nissl staining were performed on the hippocampal DG region of rats in each group to observe neuronal damage. As shown in Figure 3, in the sham group, neuronal cells were arranged neatly and in an orderly manner, with normal morphology and distinct nuclear structures. The number of cells showing death was relatively small, and there were more Nissl bodies in the neurons. The arrangement of neurons in the VCI group was disordered, with obvious structural abnormalities and nuclear damage, a higher number of cell deaths (red arrow), and a decrease in the number of Nissl bodies. Compared with the VCI group, the rosemary group and the rosemary+SIRT1 inhibitor group showed significant improvements in neuronal cell damage (red arrow), nuclear structural damage, an increased number of Nissl bodies in neurons, and overall cell damage, indicating that rosemary can alleviate neuronal damage in VCI rats.

Effect of rosemary on the ultrastructure of mitochondria in the hippocampus of VIC rats observed by transmission electron microscopy

Mitochondrial damage was observed by transmission electron microscopy. As shown in Figure 4, in the sham

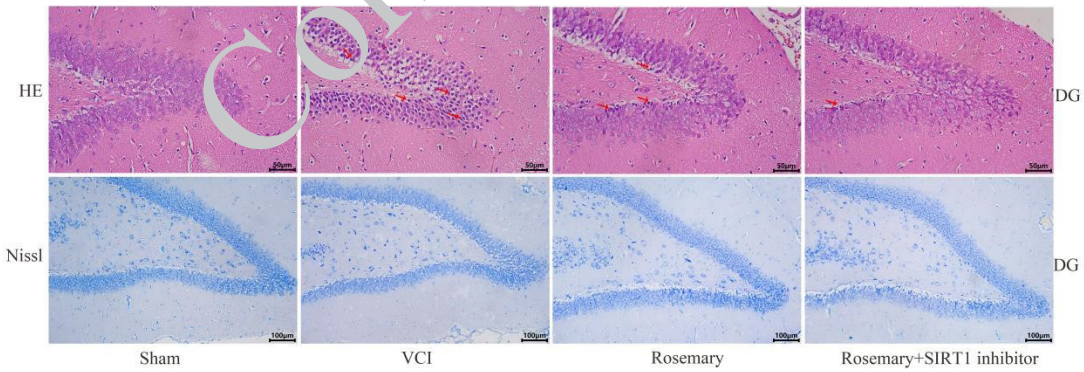

Figure 3. Results of HE and Nissl staining in the hippocampal DG region of rats in each group (scale bar =50, 100 μm; n = 3)
SIRT1: Silent mating type information regulation 1; VCI: Vascular cognitive impairment

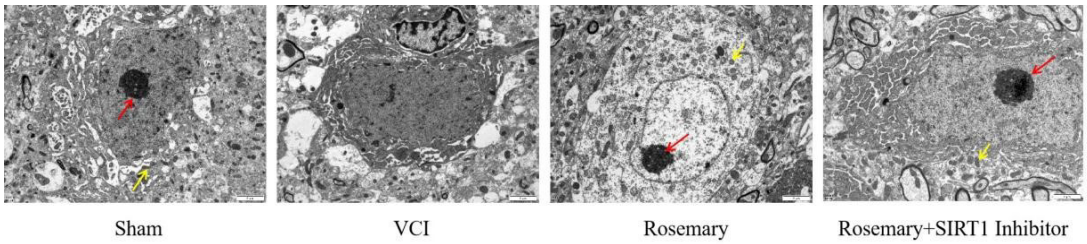

Figure 4. Rat mitochondrial damage was observed by transmission electron microscopy (scale bar = 2 μm; n = 3)
SIRT1: Silent mating type information regulation 1; VCI: Vascular cognitive impairment

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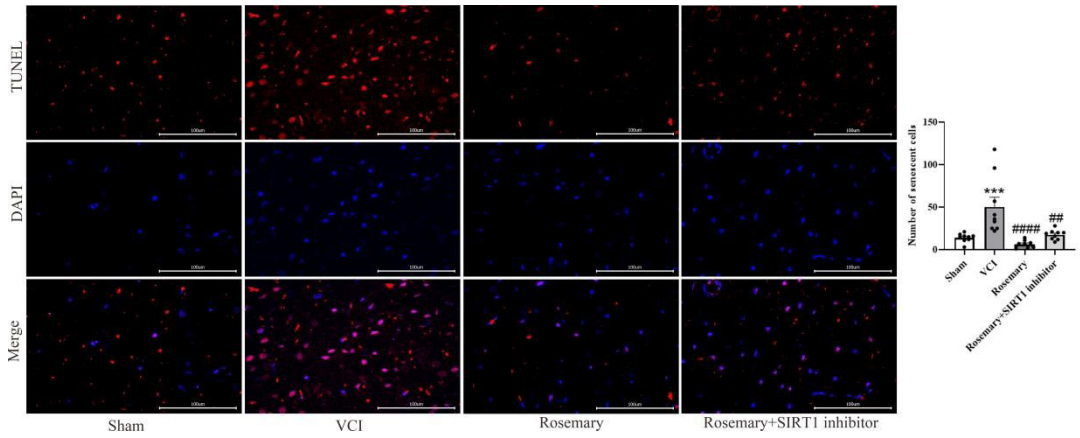

Figure 5. TUNEL staining was used to observe the apoptosis of cells in rat brain tissues
Data are presented as mean ± SD; ***P<0.001 vs Sham group; ##P<0.01, ####P<0.0001 vs VCI group; scale bar = 100 μm; n = 3; experiments were repeated three times. SIRT1: Silent mating type information regulation 1; VCI: Vascular cognitive impairment

group, the ultrastructure of cells was normal, with condensed chromatin (red arrow), abundant organelles, intact mitochondrial morphology and structure, and a high number of mitochondria with normal cristae structure and no signs of lysis (yellow arrow). In the VIC group, cell ultrastructure was disordered, with uncondensed chromatin, shrunken cells, fewer mitochondria, and cristae dissolution. In the rosemary group and the rosemary+SIRT1 inhibitor group, the ultrastructure of cells was normal, with condensed chromatin (red arrow), abundant organelles, and a higher number of mitochondria (yellow arrow). These results indicated that rosemary can reduce mitochondrial damage in the brain tissues of VCI rats.

Rosemary inhibits apoptosis in VCI rats

TUNEL staining was used to assess apoptosis in rat brain tissues. As shown in Figure 5, compared with the sham group, the VCI group showed a significant increase in the number of apoptotic cells (P<0.001). Compared with the VCI group, the rosemary group and the rosemary+SIRT1 inhibitor group showed a significant reduction in the number of apoptotic cells (P<0.0001, P<0.01), and the rosemary group had even fewer apoptotic cells. Compared with the rosemary group, the rosemary+SIRT1 inhibitor group showed an increase in the number of apoptotic cells (P>0.05), but the difference was not significant. These results suggested that rosemary could inhibit apoptosis in VCI rats.

Rosemary reduces LC3B expression in the brain tissue of VCI rats

The expression of LC3B in the rat brain tissues was detected by immunofluorescence. As shown in Figure 6, compared with the sham group, the expression of LC3B protein in the brain tissue of the VCI group was increased(P<0.0001). Compared with the VCI group, the expression of LC3B protein in the brain tissue was significantly reduced in both the rosemary group and the rosemary+SIRT1 inhibitor group, with the rosemary group showing a more significant decrease(P<0.0001). Compared with the rosemary group, the expression of LC3B protein in the brain tissue of the rosemary+SIRT1 inhibitor group was significantly increased(P<0.01). These results indicated that rosemary could reduce autophagy in the brain tissues of VCI rats, while SIRT1 inhibitors could weaken this effect.

Rosemary improves VCI by regulating SIRT1 and mTOR to inhibit cell apoptosis and autophagy

The protein expression of caspase-3, SIRT1, LC3B,

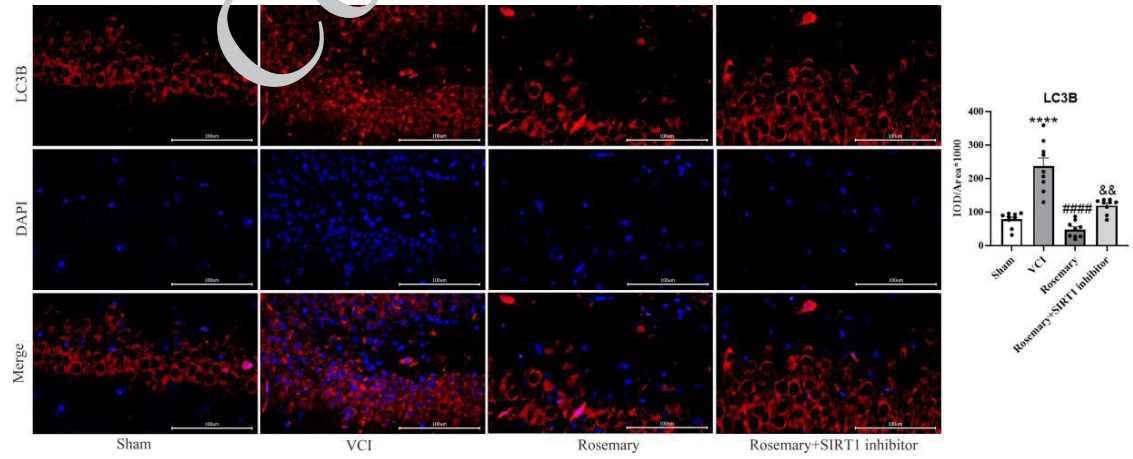

Figure 6. Expression of LC3B in the rat brain tissues was detected by immunofluorescence
Data are presented as mean ± SD; ****P<0.0001 vs the Sham group; ####P<0.0001 vs the VCI group; &&P<0.01 vs the Rosemary group. scale bar =100 μm. n=3, and the test was repeated 3 times. SIRT1: Silent mating type information regulation 1; VCI: Vascular cognitive impairment

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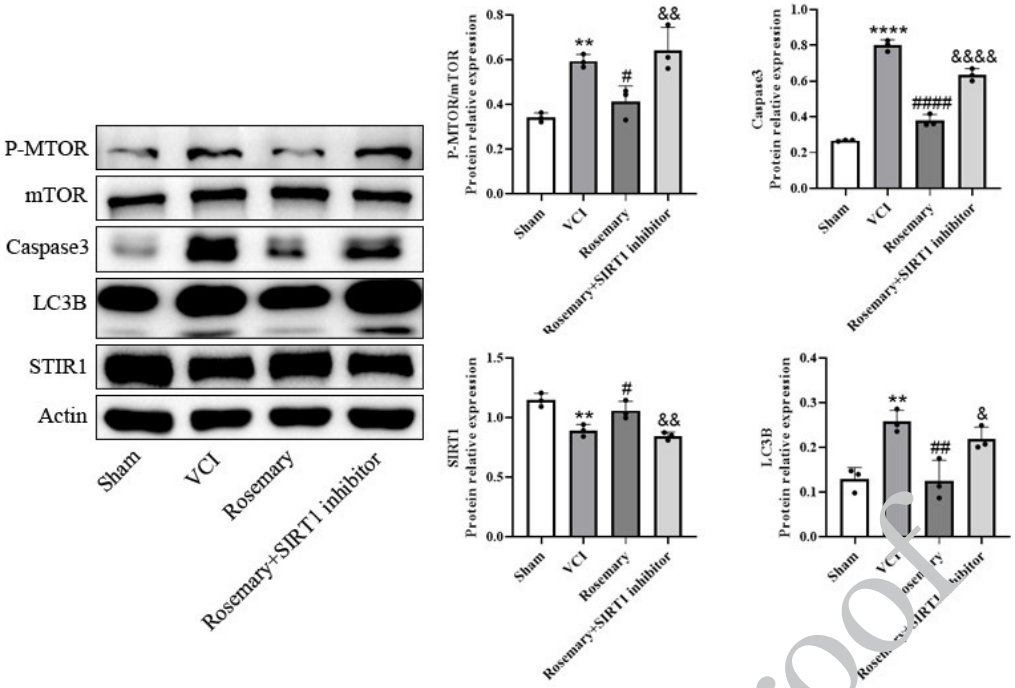

Figure 7. Protein expression of Caspase-3, SIRT1, LC3B, mTOR, and p-mTOR in rat hippocampal tissue was detected by Western blot
Data are presented as mean ± SD; ***P*<0.01,*****P*<0.0001 compared with the sham group;#*P*<0.05, ##*P*<0.01, ####*P*<0.0001 compared with the VCI group; &*P*<0.05, &&*P*<0.01, &&&&*P*<0.0001 compared with the Rosemary group, n=3.
SIRT1: Silent mating type information regulation 1; VCI: Vascular cognitive impairment

mTOR, and p-mTOR in rat hippocampal tissue was detected by western blot. As shown in Figure 7, compared with the sham group, the levels of caspase-3, LC3B, and p-mTOR/mTOR in the hippocampal tissue of the VCI group were significantly increased (*P*<0.0001, *P*<0.01, *P*<0.01), while the level of SIRT1 protein was significantly decreased (*P*<0.01). Compared with the VCI group, the rosemary group and the rosemary+SIRT1 inhibitor group showed varying degrees of decrease in caspase-3, LC3B, and p-mTOR/mTOR levels (*P*<0.0001, *P*<0.01, *P*<0.05), while the SIRT1 protein level was increased (*P*<0.05), with the rosemary group showing a more significant effect. Compared with the rosemary group, the rosemary+SIRT1 inhibitor group showed significantly increased levels of caspase-3, LC3B, and p-mTOR/mTOR (*P*<0.0001, *P*<0.01, *P*<0.05), and significantly decreased levels of SIRT1 protein (*P*<0.01). The results indicated that rosemary could inhibit cell apoptosis, autophagy, and improve VCI, while the SIRT1 inhibitor could reverse the effect of rosemary.

Discussion

VCI, as a disease recognized in recent years, covers the entire process from mild cognitive impairment to vascular dementia. Many factors affect VCI, including genetics, race, age, etc. Vascular risk factors significantly affect VCI and increase the likelihood of its occurrence (3). The number of VCI patients and the incidence rate of VCI have increased in recent years, which has become one of the major health problems in the world. However, current treatment methods for VCI primarily focus on prevention, with no specific treatments. Rosemary is a folk herb used to treat neurological diseases, and its effects have been demonstrated in AD and vascular dementia (16, 23). Essential oils are volatile mixtures extracted from plants, and studies have shown that essential oil therapy has a cognitive-improving effect. Rosemary essential oil has been reported to improve the cognitive function of AD mice (24), and it may have therapeutic effects on other neurological diseases. This study explored the effect of rosemary essential oil on VCI and the related mechanisms.

VCI can cause behavioral and cognitive disorders, while components in rosemary oil have been confirmed to ameliorate hippocampal neurotoxicity and learning and memory impairments, protect neurons, and thereby improve abnormal animal behaviors (25). In this study, a VCI animal model was constructed, and it was found that after rosemary treatment, the number of platform crossings in VCI rats increased, and escape latency decreased. Pathological findings in brain tissue showed recovery from neuronal damage, an increase in Nissl bodies, and a reduction in apoptotic neurons, indicating that rosemary can alleviate neuronal damage and inhibit neuronal apoptosis in VCI rats. The regulation of mitochondria is crucial in the process of cell apoptosis. The permeability of the mitochondrial membrane changes during the process of apoptosis activation, releasing cytochrome C to promote cell apoptosis. When mitochondria are damaged and the regulation of apoptosis is disrupted, this can lead to excessive apoptosis and tissue damage. It has been shown that mitochondrial dysfunction can lead to various types of nerve damage, with mitochondrial oxidative stress, calcium ion imbalance, and autophagy imbalance being core factors in the pathogenesis of VCI (26). The transmission electron microscopy results of this study showed that the mitochondria of VCI rats shrank, the number of mitochondria was decreased, and the mitochondrial cristae dissolved. However, after treatment with rosemary, the mitochondrial damage in VCI rats was significantly reduced, indicating that rosemary can alleviate mitochondrial damage in VCI rats.

Autophagy is the process by which cells clear damaged

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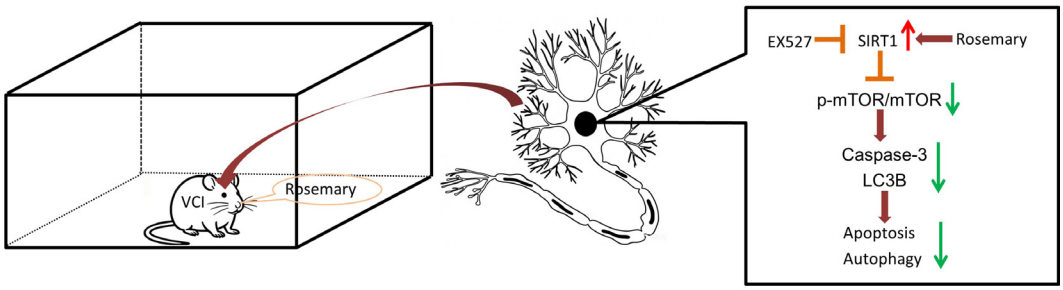

Figure 8. Proposed mechanistic scheme: Rosemary inhalation attenuates vascular cognitive impairment via SIRT1/mTOR pathwaymediated inhibition of apoptosis and regulation of autophagy
SIRT1: Silent mating type information regulation 1

proteins and organelles to maintain cellular homeostasis (27). Autophagy can ensure cell survival and reduce inflammation, whereas excessive autophagy can lead to cytoplasmic degradation and induce apoptosis or necrosis (28). Research has shown that during the induction of VCI in chronic hypoxic-ischemic brain injury, cortical and hippocampal neurons undergo excessive autophagy, leading to a decline in cognitive and learning abilities (29, 30). Moreover, it has been found that the brain undergoes autophagy overactivation during hypoxia-ischemia, and an increase in the expression of the autophagy-related marker Beclin-1 significantly exacerbates the pathological process of vascular dementia (31). Therefore, inhibiting autophagy overactivation during VCI and protecting neurons from damage may be a potential treatment for VCI. Microtubule-associated protein light chain 3 (LC3) is a key component in the formation of autophagosomes. LC3 is converted to LC3-I through cleavage, and it binds to the lipid membrane of autophagosomes through cleavage to form LC3-II. LC3 has four subtypes, namely LC3A, LC3B, LC3B2, and LC3C, which are distributed in different tissues, with LC3B being the main subtype in brain tissue (32). In this study, we conducted detection of LC3B content in the hippocampus of rats and found that the expression of LC3B was increased in VCI rats. However, after treatment with rosemary, the expression of LC3B was decreased in VCI rats, indicating that rosemary can reduce autophagy in the hippocampus of VCI rats and protect neurons from damage.

Sirtuin 1 (SIRT1) in the rat hippocampus is not only an important regulator of cellular metabolism but also implicated in neurodegenerative diseases. SIRT1 plays an important role in maintaining neurovascular integrity (33). It has been shown that elevating SIRT1 during cerebral ischemia-reperfusion can reduce mitochondrial damage, decrease neuronal apoptosis and autophagy, thereby improving cognitive impairment in patients (34). Mammalian target of rapamycin (mTOR) plays an important role in regulating cellular metabolism and growth. MTOR can directly regulate autophagy by phosphorylating Ulk1 (35), and studies have shown that SIRT1 can negatively regulate mTOR, thereby inhibiting autophagy and apoptosis. Activating the SIRT1/mTOR pathway can reduce cell apoptosis, oxidative stress, and inflammatory response, while the use of an SIRT1 inhibitor can weaken this effect (36). Caspase-3 is a key executor of cell apoptosis, and cell apoptosis is inevitable when caspase-3 is hydrolyzed into cleaved caspase-3 (37). It has been reported that caspase-3 is elevated in the brain tissue of rats with VCI, and that regulating mTOR can reduce caspase-3 expression and inhibit apoptosis (38). In this study, we found that SIRT1 expression was decreased, and the levels of caspase-3, LC3B, and p-mTOR/mTOR were increased in VCI rats. After treatment with rosemary, the expression of SIRT1 was increased, while the levels of caspase-3, LC3B, and p-mTOR/mTOR were decreased in VCI rats. However, the use of a SIRT1 inhibitor attenuated rosemary's therapeutic effect, indicating that SIRT1 inhibition can weaken rosemary's therapeutic effect on VCI. This demonstrates that the therapeutic effect of rosemary on VCI may be achieved by regulating SIRT1 expression and inhibiting cell apoptosis and autophagy.

Although this study revealed that rosemary can inhibit apoptosis and autophagy and improve cognitive impairment in VCI rats by regulating the SIRT1/mTOR pathway, there are still limitations. First, this study used SD rats to simulate VCI without testing clinical samples, which cannot fully reflect the symptoms of VCI patients. Second, the safety and efficacy of rosemary essential oil in patients remains to be verified. Finally, in combination, the therapeutic effects of rosemary essential oil on VCI may not be limited to SIRT1 regulation. In future studies, we will further explore the underlying mechanisms by which rosemary essential oil improves VCI, providing a theoretical basis for its use as a therapeutic strategy for VCI in clinical practice.

Conclusion

This study demonstrates that rosemary inhalation can alleviate cognitive and behavioral abnormalities in VCI rats by regulating apoptosis and autophagy via the SIRT1/mTOR signaling pathway (Figure 8). At present, studies on the effects of rosemary inhalation in VCI are rare. This study fills a gap in this field and reveals a novel mechanism by which rosemary essential oil improves VCI, providing a theoretical basis for its future clinical application in VCI treatment.

Acknowledgment

This work was supported by the Scientific Research Project of the Education Department of Jiangxi Province (No. GJJ2200945).

Authors' Contribution

F S designed the experiments, prepared the draft manuscript, and created the visualizations; L W and W Y performed experiments and collected data; Ensi Hong discussed the results and strategy; J B and HW revised the article critically or edited it; Y Z supervised, directed, and managed the study; F S, E H, J B, W Y, L W, H W, and Y Z approved the final version for publication.

Corrected Proof

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We acknowledge the use of Doubao AI to generate images of rats and neurons in Figure 8.

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