Asiaticoside reduces autophagy and improves memory in a rat model of dementia through mTOR signaling pathway regulation

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Abstract. Vascular dementia (VD) is one of the leading causes of neurological disorder following Alzheimer's disease. The present study evaluated the possible role of asiaticoside in the treatment of rats with VD and its inhibitory effects on autophagy in hippocampal tissues. Double ligation was used for permanent occlusion of the arteries, and spatial memory was assessed using the T-maze test. Western blotting was used for determination of protein expression levels and H&E staining for histological analysis. Treatment of rats with VD with asiaticoside significantly alleviated the impairment in spontaneously altered behaviors and significantly reduced escape latency. VD mediated a decrease in distance travelled, swim time and number of platform crossings, whereas this was alleviated by asiaticoside. Furthermore, VD-mediated hippocampal tissue damage was significantly alleviated by asiaticoside treatment ($P<0.05$), and asiaticoside alleviated formation of autophagosomes and markedly suppressed the number of primary lysosomes. In asiaticoside-treated rats, VD-mediated increases in Beclin 1 and microtubule-associated protein light chain 3 (LC3) II expression in the hippocampal tissues were alleviated. Asiaticoside treatment also prevented suppression of mammalian target of rapamycin (mTOR) phosphorylation in VD rat hippocampal tissues. Notably, the rapamycin-mediated suppression of phosphorylated-mTOR, and elevation of Beclin 1 and LC3II expression in the rat hippocampus could not be alleviated by asiaticoside treatment. In conclusion, asiaticoside effectively prevented cerebral ischemia-mediated cognitive impairment and neuronal damage in the rats. Moreover, autophagy was inhibited and the mTOR pathway was activated in rats with cerebral ischemia by asiaticoside treatment. Therefore, asiaticoside may warrant further study as a therapeutic agent for the treatment of dementia.

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Introduction

Vascular dementia (VD) is one of the leading causes of neurological disorder following Alzheimer's disease and accounts for 15% of patients with neurological disorders worldwide (1). Patients with VD suffer from memory loss and cognitive impairment, which progressively worsen over the time (2). Numerous vascular risk factors have been identified that are associated with the development of VD and its progression (3). It has been suggested that VD may affect more individuals in the future as the population ages, and the average survival of patients following stroke and cardiovascular disorders increases (4). The symptoms of VD include cognitive impairment and a reduction in memory. Currently, several compounds including, memantine, galantamine, donepezil and rivastigmine, are undergoing clinical trials for treatment of VD but the obtained results are not satisfactory. Thus, the development of effective treatment for VD is urgently needed to prevent neurological damage in more individuals.

Autophagy is the cellular process leading to self-degradation that regulates stability in the body environment by eliminating damaged cellular components, such as mitochondria (5,6). Increased autophagy causes cell death via self-digestion, and degrades cellular organelles and proteins (7). The ischemia-induced activation of autophagy leads to neuronal damage in brain tissues (8,9). This suggests that autophagy may have a vital role in inducing neuronal damage to the central nervous system in patients with ischemia. Mammalian target of rapamycin (mTOR) serves a leading role in the regulation of cellular growth, survival, protein translation and autophagy (10). A variety of autophagic cellular processes are controlled by phosphorylated (p)-mTOR/mTOR signaling pathways (11). Microtubule-associated protein light chain 3 (LC3) II is an autophagy-related factor, which has been widely studied as an autophagic protein (12). LC3II serves an important role in the development of autophagosomes and their maturation, and is therefore used for monitoring the autophagic activity in cells (12).

Naturally obtained compounds from diverse sources have demonstrated potential pharmacological activities in multiple

diseases. Asiaticoside is a saponin compound with a monomeric structure that is obtained from the medicinal plant *Centella asiatica*. Pharmacological screening has identified various properties of asiaticoside, including hepatoprotective (13), antioxidant (14), neuroprotective (15) and anti-inflammatory (16) activities. In addition, numerous saponins have been shown to possess a diverse range of pharmacological activities; notably, some saponins are in the clinical trials stage and a few have been approved as therapeutic drugs by the Food and Drug Administration (14,16). Additionally, saponins exhibit their activity through multiple pathways in different types of diseases/disorders. The present study evaluated the potential role or asiaticoside (Fig. 1) in the treatment of a rat model of VD and its inhibitory effects on autophagy in hippocampal tissues.

Materials and methods

Animals and grouping. A total of 50 male Sprague-Dawley rats (age, 8 weeks; weight, 240-270 g) were obtained from the Animal Center, Shenyang Medical University. The rats were individually maintained in sterile cages in an animal center under ~55% humidity and at 23±2˚C, and were exposed to 12-h light/dark cycles. All rats were allowed free access to water and a laboratory rodent diet. The experimental procedures involving rats were conducted in accordance with the guidelines of the Care and Use of Laboratory Animals Committee China Medical University (15). The present study was approved by the Animal Ethics Committee, Dongying District People's Hospital (Dongying, China; approval. no. DSH/2017/067). The rats were separated into five groups (n=10/group): Sham group, VD group, VD + asiaticoside group, rapamycin group and rapamycin + asiaticoside group. The rats in all groups, with the exception of the sham group, were subjected to bilateral occlusion of carotid arteries (17). Chloral hydrate (10%; 300 mg/kg) anesthesia was intraperitoneally administered to the rats, after which they were fixed supine on hot pads and a ventral midline incision was made to the neck. No signs of peritonitis were observed in the rats following anesthesia with 10% chloral hydrate. Muscles on either side of the trachea were incised carefully to expose the carotid arteries. Subsequently, double ligation was performed for permanent occlusion of the arteries. The same procedure without vessel ligation was repeated in the sham group. The rapamycin and rapamycin + asiaticoside groups were injected with rapamycin (50 μl) 1 day before surgery directly into the ventricle using a catheter. Asiaticoside dissolved in physiological saline at 5 mg/kg body weight was given to treatment groups via the intragastric route as a single dose after surgery.

Behavioral assessment using T-maze tests. The spatial memory of rats was assessed using the T-maze test 28 days after surgery, using previously reported methodology (18). Each trial in the T-maze test involves two runs: A sample run and a choice run. The sample run consisted of forcing the rats to enter one of the two arms of the maze in order to obtain sugar placed in left arm, while the second arm of the maze was closed using a sliding door. During the choice run, the door was opened and rats were left to choose one of the arms freely. The time duration set between the two runs was 10 sec, and the rats entering the previously unvisited arm were rewarded. Subsequently, the time duration between the two runs was increased to 90 and 180 sec. Each session involved five trials every day and the time gap between two trials was set at 10 min. The number of corrections was taken as the number of times rats entered the arm that was previously unvisited.

Morris water maze (MWM) test. The MWM test was used to assess cognitive ability of the animals 28 days after surgery (17). Briefly, the rats were given four training trials every day for 5 consecutive days. The training trials consisted of placing the rats alternately in four different quadrants of a pool of water and allowing them to locate a platform during 120 sec. The rats were then given 20 sec to rest on the platform; time taken to find the platform was counted as escape latency. The rats unable to locate the platform during the assigned duration were guided towards it and allowed to rest for 20 sec. The platform was removed from the pool on the 6th day of the probe test and rats were permitted to swim freely to locate the removed platform. The swimming activity of the rats was monitored and recorded video-graphically. The platform crossings were recorded by calculating the platform location crossed by each rat.

Extraction of brain tissues. The rats were sacrificed using pentobarbital overdose. Subsequently, normal saline and paraformaldehyde (4%) in sodium phosphate buffer were perfused transcardially. The rat brains were dissected and then subjected to fixing in 4% paraformaldehyde at 4˚C. After 3 days of fixing, the brain samples were embedded in paraffin and sliced into 2-μm sections.

Transmission electron microscopy (TEM). The brain tissues were fixed with 2.5% glutaraldehyde in PBS at 4˚C for 2 h, and then with 1% osmium tetroxide (Ph 7.4) for 2.5 h at 4˚C. Subsequently, the tissues were stained with uranyl acetate (1% aqueous) solution overnight at 4˚C prior to embedding in Durcupan (Sigma-Aldrich; Merck KGaA). An ultracut microtome (Leica Microsystems, Inc.) was used to cut hippocampal tissues into ~60-nm sections, which were placed on formvar-coated copper grids. The sample sections were subjected to staining for 5 min with uranyl acetate (2.5%) and lead citrate (3%) followed by examination under a 7650 transmission electron microscope (Hitachi High-Technologies Corporation).

Western blotting. The rats were sacrificed using pentobarbital overdose. Subsequently, normal saline and 4% paraformaldehyde in sodium phosphate buffer was perfused transcardially. The hippocampal tissues were excised and then homogenized with RIPA lysis buffer (Beyotime Institute of Biotechnology) mixed with PMSF. The lysates were centrifuged at 12,000 x g for 15 min at 4˚C, followed by protein content determination using the BCA assay kit (Beyotime Institute of Biotechnology). The protein samples (30 μg) were separated by SDS-PAGE on 12% gels and were then transferred to PVDF membranes, which were blocked using 3% BSA (Sigma-Aldrich; Merck KGaA) in Tris-buffered saline for 40 min at room temperature. The membranes were probed with primary antibodies at 4˚C overnight, washed with PBS and then incubated for 2 h

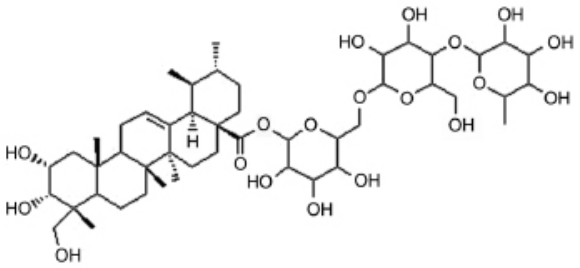

Figure 1. Chemical structure of asiaticoside.

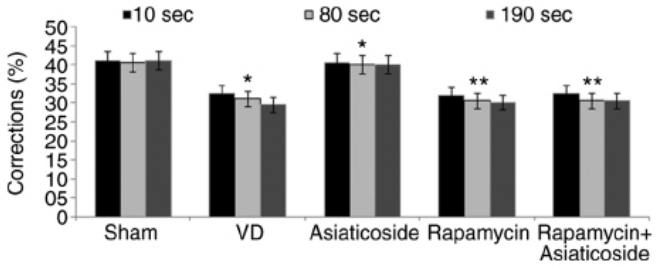

Figure 2. Effect of asiaticoside on spatial memory in a rat model of VD. The improvement in spontaneously altered behavior impairments was analyzed in the VD, sham, asiaticoside, rapamycin and rapamycin + asiaticoside groups using T-maze tests. The time intervals between two runs were 10, 80 and 190 sec. *P<0.05 and **P<0.01 vs. sham group. VD, vascular dementia.

with horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) at room temperature. Detection and visualization of protein bands were performed using an enhanced chemiluminescence system (EMD Millipore) and the blots were semi-quantified by Quantity-One software version 4.6.3 (Bio-Rad Laboratories, Inc.). The primary antibodies used were: Anti-mTOR (cat. no. 2972; dilution 1:400; Cell Signaling Technology, Inc.), anti-LC3B (cat. no. 2775; dilution 1:1,000; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-mTOR (cat. no. 2971; dilution 1:250; Cell Signaling Technology, Inc.), anti-Beclin 1 (cat. no. sc-48341; dilution 1:1,000; Santa Cruz Biotechnology, Inc.) and anti-β-actin (cat. no. 4967; dilution 1:1,000, Cell Signaling Technology, Inc.).

Nissl staining. The rats were sacrificed after anaesthetization with overdose of sodium pentobarbital. Transcardial perfusion was performed with normal saline (0.9%) and then with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). Dissection of the brain was followed by fixing with 4% paraformaldehyde at 4˚C for 3-days and then paraffin embedding. Brains were sliced into 5-μm sections followed by staining at 60˚C for 45 min with 1% Toluidine Blue. Neuronal survival was determined by the examination of Nissl-positive cells in the rat hippocampus. Light microscope (model, BX53; Olympus Corporation) was used for calculation of normal neurons in the CA1 subfield at x400 magnification. The average number of neurons was calculated in three sections and five representative fields were randomly chosen for each section.

Histological analysis using H&E staining. The rats were sacrificed after anaesthetization with 200 mg/kg sodium pentobarbital intraperitoneally. Hippocampal tissues were extracted, subjected to PBS washing and then fixed in 10% neutral buffered formalin at room temperature for 45 min. The tissues were decalcified using EDTA (10%) followed by embedding in paraffin and were then sliced into 3-μm sections. The sections were subjected to H&E staining for 40 min at room temperature and were then examined under a light microscope (Olympus IX81; Olympus Corporation) at x200 magnification.

Statistical analysis. The data are presented as the mean ± standard deviation of three experiments. Data analysis was performed using SPSS 16.0 statistical software (SPSS, Inc.). The statistical differences between groups were determined using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Asiaticoside improves cognitive function in a rat model of VD. Changes in behaviors were significantly impaired in the VD rat model group compared with in the sham group (Fig. 2). Asiaticoside treatment of rats with VD significantly improved the changes in behaviors. In addition, rapamycin administration also mediated an impairment in spontaneously altered behaviors in the rats. Asiaticoside failed to significantly alleviate spontaneously altered behavior impairment in VD rats treated with rapamycin. These findings indicated that asiaticoside effectively improved VD-mediated changes in behavior compared with the VD group.

Asiaticoside shortens escape latency in a rat model of VD. Escape latency exhibited a significant increase in the VD model group compared with in the sham group (P<0.05; Fig. 3). Treatment of VD rats with asiaticoside caused a significant reduction in escape latency (P<0.05). Rapamycin administration also increased escape latency. The rapamycin-mediated increase in escape latency in rats could not be significantly reduced by asiaticoside treatment.

Asiaticoside increases swimming time in a rat model of VD. The distance travelled, swim speed, swim time and number of platform crossings were significantly reduced for rats in the VD group compared with in the sham group (P<0.05; Fig. 4). However, asiaticoside significantly alleviated VD-mediated decreases in distance travelled, swim time and number of platform crossings (P<0.05). Administration of rapamycin also significantly reduced distance travelled, swim time and number of platform crossings (P<0.05). However, asiaticoside treatment could not significantly alleviate the rapamycin-mediated reduction in distance travelled, swim time and number of platform crossings.

Asiaticoside promotes neuronal survival in rats with VD. Abnormalities in the hippocampal tissues of rats were detected using H&E staining (Fig. 5A) and analysis of Nissl bodies (Fig. 5B). The abnormalities were markedly evident in the hippocampus of VD rats compared with in the sham group. Neuronal apoptosis and degeneration were clearly detected in the hippocampus of VD rats. Conversely, VD-mediated hippocampal tissue damage was significantly alleviated by asiaticoside treatment of the rats (P<0.05). In addition,

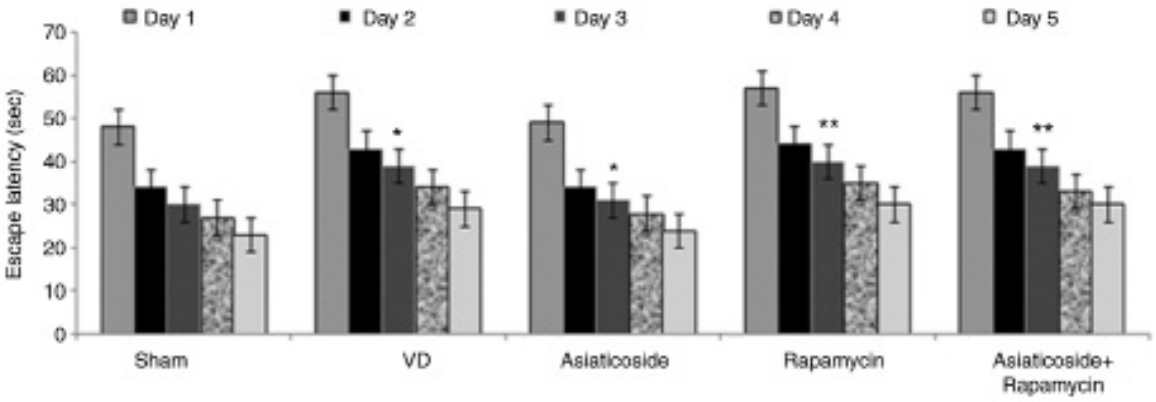

Figure 3. Effect of asiaticoside on escape latency in a rat model of VD. Escape latency was measured in the VD, sham, asiaticoside, rapamycin and rapamycin + asiaticoside groups using Morris water maze test. The tests were conducted on days 1, 2, 3, 4 and 5 post-ischemia. *P<0.05 and **P<0.01 vs. sham group. VD, vascular dementia.

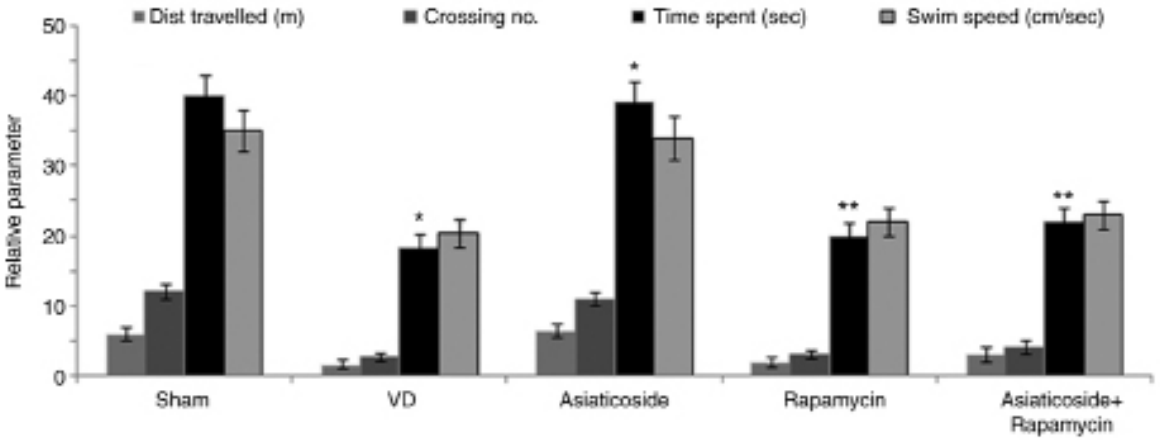

Figure 4. Effect of asiaticoside on cognition in VD rats. The distance travelled, swim time, number of platform crossings and swim speed were measured in the VD, sham, asiaticoside, rapamycin and rapamycin + asiaticoside groups using Morris water maze test. *P<0.05 and **P<0.01 vs. sham group. VD, vascular dementia; Dist, distance.

abnormalities were also induced in the hippocampal tissues of rats treated with rapamycin. However, no significant improvement in rapamycin-induced hippocampal tissue damage was observed following treatment with asiaticoside.

Asiaticoside inhibits autophagosome formation in the hippocampus of rats with VD. Autophagosome formation was clearly detected near the nuclei in the hippocampus of rats in the VD group, but was absent in the sham group (Fig. 6A and B). The primary autophagosome count was also increased in the hippocampus of rats with VD. Treatment of rats in the VD group with asiaticoside alleviated formation of autophagosomes and markedly suppressed the number of primary lysosomes. In rapamycin-administered rats, the numbers of autophagosomes were markedly increased. No significant reduction in autophagosome formation was observed in rapamycin-administered rats treated with asiaticoside.

Asiaticoside upregulates p-mTOR expression and suppresses autophagy-related proteins in a rat model of VD. The expression levels of Beclin 1 and LC3II were significantly elevated in the hippocampal tissues of rats in the VD group compared with in the sham group (Fig. 7A and B). Treatment with asiaticoside alleviated VD-mediated increases in Beclin 1 and LC3II expression in the rat hippocampal tissues. The phosphorylation of mTOR in the hippocampal tissues of rats in the VD group was markedly suppressed compared with in the sham group. Conversely, asiaticoside treatment prevented suppression of mTOR phosphorylation in the hippocampal tissues of rats with VD.

Asiaticoside inhibits autophagy in rats with VD via the mTOR pathway. Rapamycin administration markedly suppressed activation of mTOR in rat hippocampal tissues compared with in the sham group (Fig. 8). Conversely, the protein expression levels of Beclin 1 and LC3II were markedly upregulated by rapamycin in the hippocampus of rats. The rapamycin-mediated suppression of p-mTOR, and elevation of Beclin 1 and LC3II expression in the rat hippocampus could not be alleviated by asiaticoside treatment.

Discussion

VD is a commonly diagnosed type of dementia, which is generally caused by cerebrovascular diseases and is associated

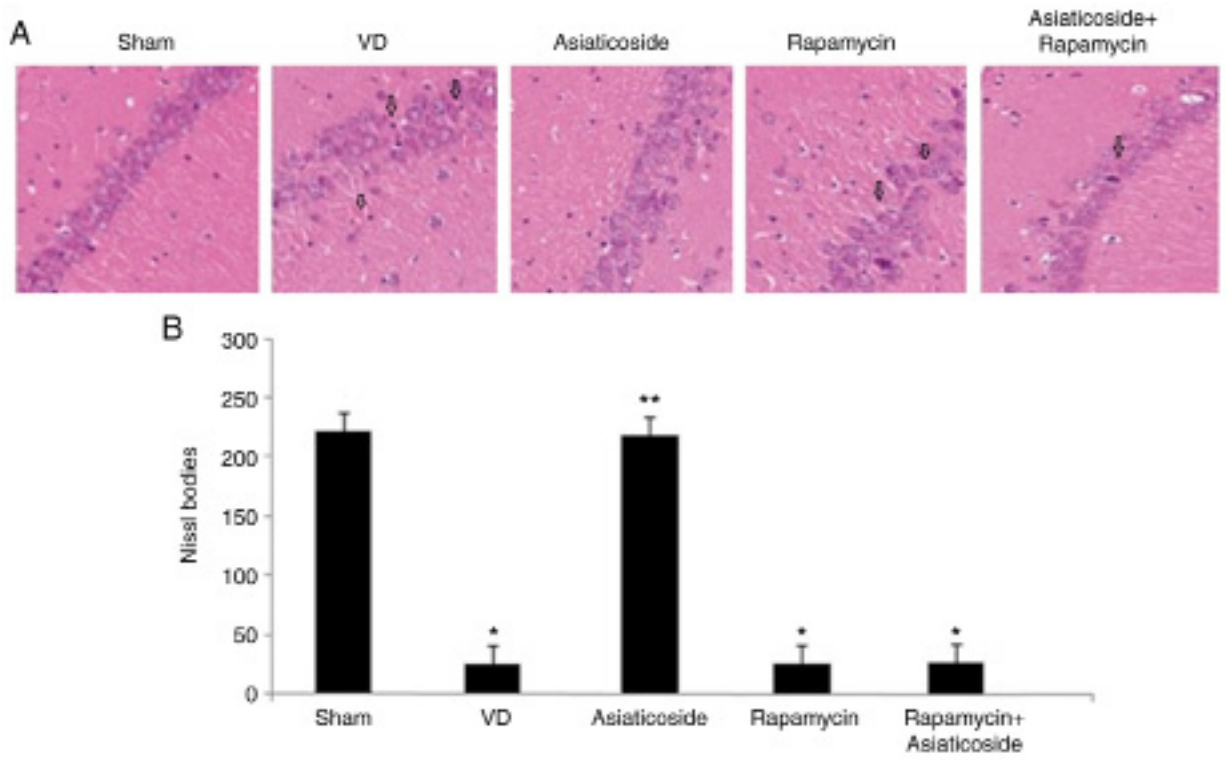

Figure 5. Effect of asiaticoside on hippocampal damage in rats with VD. (A) Effects of asiaticoside on neuronal damage induced by VD and rapamycin in the rat were detected by Nissl staining staining. Arrows indicate neuronal damage. Magnification, x200. (B) Nissl bodies in the rat hippocampus. *P<0.05 and **P<0.01 vs. sham group. VD, vascular dementia.

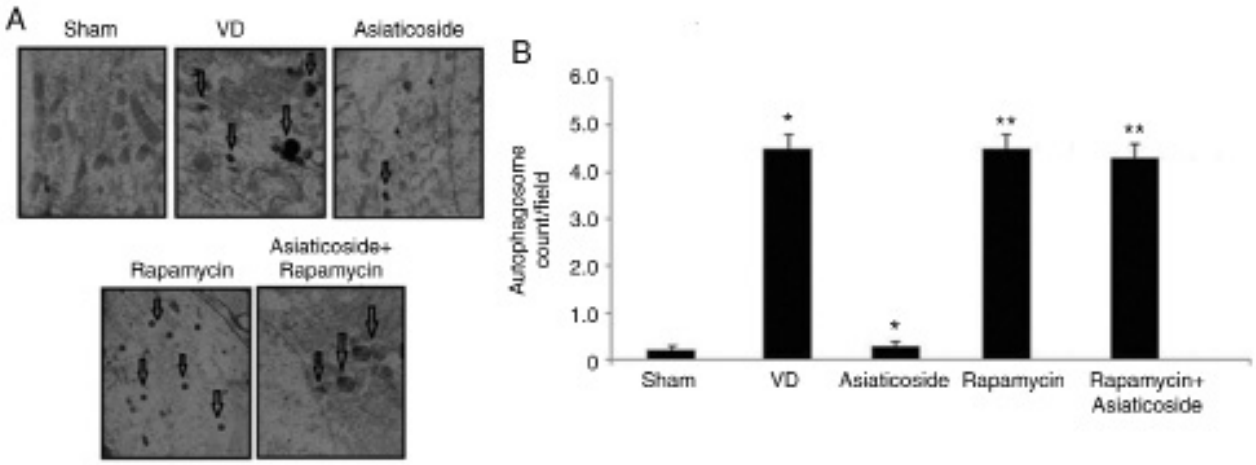

Figure 6. Effect of asiaticoside on autophagosomes in the hippocampus of rats with VD. (A) Effects of asiaticoside on autophagosome formation induced by VD and rapamycin in the rat hippocampus were detected by electron microscopy. Arrows indicate autophagosomes formed. Magnification, x2,500. (B) Semi-quantification of autophagosome formation in the rat hippocampus. *P<0.05 and **P<0.01 vs. sham group. VD, vascular dementia.

with several other factors, including smoking, high blood pressure and diabetes (19). The pathological mechanism of VD is not clearly known; therefore, studies are urgently required to develop effective treatments. Autophagy is a cellular adaptive process, which is induced by various stress factors in multi-cellular organisms and is associated with dynamic catabolism (20). Excessive autophagy leads to cell death and contributes significantly to ischemia-induced neuronal damage (21,22). Previous studies reported that neurological impairments caused by cerebral ischemia were modulated by targeting autophagy induction (23,24). Autophagy induction is regulated by mTOR kinase, and it has been reported that autophagy may be suppressed by mTOR activation and enhanced by inhibition of mTOR (25). Autophagy is specifically induced in cells by administration of rapamycin (mTOR inhibitor), which directly inhibits mTOR (26). In addition, hippocampal neuronal damage caused by hypoxia-mediated injury has been reported to be protected by downregulation of the mTOR pathway (27). The present study established a rat model of VD using the reported protocol, and evaluated the effects of asiaticoside treatment on cognitive memory improvement using MWM and T-maze tests (17). The data

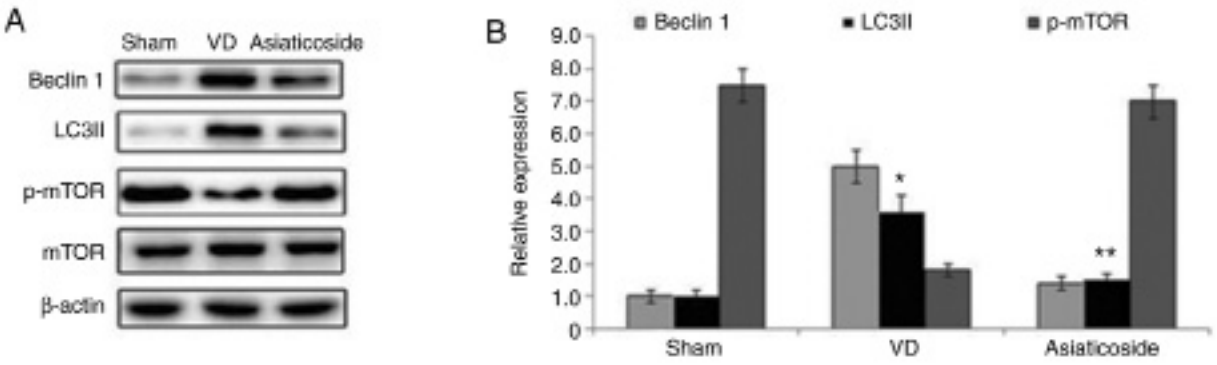

Figure 7. Effect of asiaticoside on the expression levels of autophagic proteins. (A) p-mTOR, Beclin 1 and LC3II expression levels were detected in the hippocampal tissues of rats in the sham, VD and asiaticoside groups by western blotting. (B) Semi-quantification of protein expression in the rat hippocampus. *P<0.05 and **P<0.01 vs. sham group. VD, vascular dementia; LC3II, microtubule-associated protein light chain 3 II; p-, phosphorylated; mTOR, mammalian target of rapamycin.

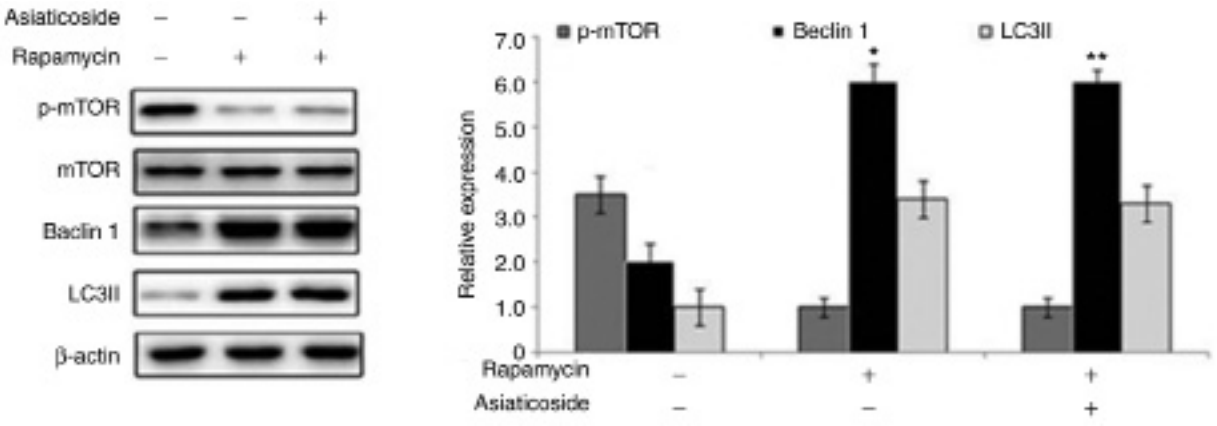

Figure 8. Effect of asiaticoside on the mTOR pathway. p-mTOR, Beclin 1 and LC3II expression levels were detected in the hippocampal tissues of rats following treatment with asiaticoside or rapamycin by western blotting. Semi-quantification of protein expression in the rat hippocampus. *P<0.05 and **P<0.01 vs. sham group. VD, vascular dementia; LC3II, microtubule-associated protein light chain 3 II; p-, phosphorylated; mTOR, mammalian target of rapamycin.

revealed that asiaticoside effectively prevented VD-mediated cognitive memory impairment in rats. However, asiaticoside was ineffective against cognitive impairment induced by VD in rats administered with rapamycin (autophagy agonist). In rats with VD, asiaticoside treatment prevented neuronal damage, as evidenced by a marked increase in Nissl-positive cell proportion compared with in the VD group. Conversely, the neuronal damage in VD rats administered with rapamycin could not be prevented by treatment with asiaticoside. These findings suggested that asiaticoside prevented VD-induced neuronal damage and cognitive impairment in rats, but could not alleviate the effect of rapamycin. Thus, asiaticoside inhibits induction of autophagy in rats to prevent VD-induced damage to the neurons.

Autophagy is associated with the transport of denatured intracellular proteins, senescent proteins and damaged organelles to lysosomes, where degradation and digestion takes place. Autophagy is a cellular defense mechanism against adverse environmental conditions encountered during various pathological processes. Previous studies have demonstrated that, in cardiomyocytes, ischemia and hypoxia induced activation of autophagy via promotion of mTOR and LC3II expression (28,29). Cardiomyocytes are protected by an appropriate level of autophagy; however, increased autophagy during ischemia and hypoxia may result in injury to myocardial cells (30). Exposure of myocardial cells to extreme levels of ischemia has been shown to lead to increased autophagy and may promote apoptosis during reperfusion (31). In addition, it has been suggested that ischemia or hypoxia may induce autophagy, which could subsequently lead to neuronal death (32-35). The present study revealed that VD induced formation of autophagosomes and enhanced the number of lysosomes in cells. TEM examination exhibited an absence of autophagosomes and significantly reduced count of lysosomes in rats with VD treated with asiaticoside. The inhibition of VD-mediated autophagy activation by asiaticoside was also confirmed by western blotting. In VD rats, the protein expression levels of LC3II and Beclin-1 were markedly higher compared with in the sham group. By contrast, treatment of rats in the VD group with asiaticoside alleviated VD-mediated upregulation of LC3II and Beclin-1 expression. These data indicated that asiaticoside may prevent VD-mediated neuronal damage via inhibition of autophagy. The effect of asiaticoside on mTOR, which is a downstream executer of autophagy, was also evaluated in rats with VD. The present study demonstrated that asiaticoside treatment markedly promoted phosphorylation of mTOR in the hippocampal tissues of VD rats; however,

in rats with VD administered with rapamycin, asiaticoside could not inhibit the induction of autophagy.

In conclusion, asiaticoside may effectively prevent cerebral ischemia-mediated cognitive impairment and neuronal damage in rats. Moreover, autophagy was inhibited and the mTOR pathway was activated in rats with VD treated with asiaticoside. Therefore, asiaticoside may be studied further as therapeutic agent for the treatment of dementia.

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Availability of data and materials

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

Authors' contributions

MG, JX and SW conducted the experimental work, performed the literature survey and analyzed the data. BD designed the study and wrote the manuscript. All authors read and approved the final manuscript. MG and BD confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee, Dongying District People's Hospital (Dongying, China; approval. no. DSH/2017/067).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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