

Asiaticoside reduces autophagy and improves memory in 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 disorders following Alzheimer's disease. The present study evaluated asiaticoside for possible role in treatment of VD in the rat model and inhibition of autophagy in hippocampus tissues. Asiaticoside treatment of the VD rats significantly ($P < 0.05$) alleviated the impairment in spontaneously altered behaviors and significantly ($P < 0.05$) reduced escape latency. VD mediated decrease in distance travelled, swim time and count of platform crossings was significantly ($P < 0.02$) alleviated by asiaticoside. The VD mediated hippocampus tissue damage was alleviated significantly ($P < 0.05$) by asiaticoside treatment. Treatment of the VD rats with asiaticoside alleviated formation of autophagosome and markedly suppressed count of primary lysosomes. In asiaticoside treated rats VD mediated increase in Beclin-1 and Microtubule-associated protein light chain 3II (LC3II) expression in the hippocampus tissues was alleviated. Asiaticoside treatment prevented suppression of mammalian target of rapamycin (mTOR) phosphorylation in VD rat hippocampus tissues. The rapamycin mediated suppression of p-mTOR and elevation of Beclin 1 and LC3II expression in rat hippocampus could not be alleviated by asiaticoside treatment. In summary, asiaticoside effectively prevents cerebral ischemia mediated cognitive impairment and neuronal damage in the rats. Moreover, autophagy was inhibited and mTOR pathway activated in rats with cerebral ischemia by asiaticoside treatment. Therefore, asiaticoside may be studied further as therapeutic agent for treatment of dementia.

Introduction

Vascular dementia (VD) is one of the leading causes of neurological disorders following Alzheimer's disease and accounts for about 15% of patients (O'Brien and Thomas 2015). The patients with VD suffer from memory loss and cognitive impairment progressively over the time (Smith 2017). Studies have found multiple vascular risk factors associated with the development VD and its progression (Jayant and Sharma 2016). It is believed that VD may influence more people in future as population ages and average survival of patients following stroke and cardiovascular disorders increases (Levine and Langa 2011). The symptoms of VD include cognitive impairment and reduction in memory of the people. The VD treatment available currently is ineffective and the mechanism of its pathogenesis is not clear. Thus, development of VD treatment is urgently needed to prevent the neurological damage in more people.

Autophagy is the cellular process leading to self-degradation and regulates stability in the body environment by eliminating damaged components of the cells like mitochondria (Voigt and Pöggeler 2013; Bharadwaj et al. 2012). The higher expression of autophagy causes death of cells by self-digestion and degrades cellular organelles and proteins (Ouyang et al. 2012). The ischemia induced activation of autophagy leads to damage of neurons in the tissues of brain (Lu et al. 2014; Fujita et al. 2015). This suggests that autophagy plays vital role in inducing neuronal damage to the central nervous system in ischemia patients. Mammalian target of rapamycin (mTOR) plays a leading role in regulation of cellular growth, survival, protein translation and autophagy (Hwang et al. 2017). A variety of autophagic cellular processes are controlled by phosphorylated (p)mTOR/mTOR signaling pathways (Chang et al. 2017).

The LC3II is one of the autophagy-related factors which has been most widely studied as autophagic protein (Schaaf et al., 2016). It serves main role in the development of autophagosomes and maturation and is therefore used for monitoring the autophagic activity in cells (Schaaf et al., 2016).

Naturally obtained compounds from diverse sources have demonstrated potential pharmacological activities multiple diseases. Asiaticoside, a compound having saponin monomeric structure is obtained from the medicinal plant *Centella asiatica*. Pharmacological screening found various properties of asiaticoside like hepato-protective (Dong et al. 2004), antioxidant (Guo et al. 2004), neuroprotective (Chen et al. 2014) and antiinflammatory (Yun et al. 2008) activities. Many of the saponins are also known for diverse range of pharmacological activities, some are in clinical trials stage and few FDA approved drugs. Additionally, saponins exhibit their activity through multiple pathways in different types of diseases/disorders. The present study evaluated asiaticoside (Fig. 1) for possible role in the treatment of VD in the rat model and inhibition of autophagy in hippocampus tissues.

Materials And Methods

Animals and grouping

Fifty male Sprague-Dawley rats (8-week old) weighing 240-270 g were obtained from the Animal Center belonging to Shenyang Medical University, China. The rats were kept in sterile cages individually in animal center under ~ 55% humidity and at $23 \pm 2^\circ\text{C}$ and were exposed to 12 h light/dark cycles. All rats were allowed to access water and laboratory rodent diet freely. The experimental procedures involving rats were conducted in accordance with the guidelines of Care and Use of laboratory animals Committee China Medical University. The approval for study was given by Ethics Committee of China Medical University. The rats were separated into five groups: sham operation (Sham), Vascular dementia alone (VD), VD + asiaticoside, rapamycin alone and rapamycin + asiaticoside groups. The rats in four groups except sham group were subjected to bilateral occlusion of carotid arteries (2-VO) (Xing et al. 2016). Chloral hydrate anaesthesia was intraperitoneally given to the rats followed by fixed supine on hot pads with a ventral midline incision in the neck. Muscles on either side of the trachea were incised carefully to expose the carotid arteries. Then double ligation was performed for permanent occlusion of the arteries. The same procedure except vessel ligation was repeated in the sham rat group. The rapamycin and rapamycin + asiaticoside groups were injected rapamycin (50 μl) one day before surgery directly into the ventricle using catheter. Asiaticoside at 5 mg/kg body weight was given to treatment groups via intragastric route as single dose after surgery.

Behavioral assessment using T-maze tests

The spatial memory of rats was assessed using T-maze test after 28th day of surgery using previously reported methodology (Deacon and Rawlins 2006). Each of the trial in T-maze test involved two runs-one sample and second choice run. Sample run consisted of forcing the rats to enter any of the two arms for getting sugar while second arm of maze was closed using a sliding door. During choice run the door was opened and rats were let to choose any of the arms freely. The time duration set between the two runs

was of 10 sec and the rats entering unvisited arm were rewarded. Subsequently the time duration between the two runs were increased to 90 and 180 sec. Each session involved five trials every day and the time gap between two trials was set to be 10 min. The count of corrections was taken as the number of times rats entered the arm which was previously unvisited.

Morris water maze (MWM) test

The MWM test was used for assessment of cognitive ability of the animals after 28 days of surgery (Xing et al. 2016). Briefly, the rats were given four trials of training session every day for five consecutive days. The training trials consisted of placing the rats alternately in four different quadrants of water pool and allowing them to locate the platform during 120 sec. The rats were then given 20 sec to rest on the platform and time taken to find the platform was counted as escape latency. The rats unable to locate the platform during assigned time duration were guided towards it and allowed to rest for 20 sec. The platforms in the water pool were removed on 6th day of probe test and rats were permitted to swim freely for locating 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.

Analysis of neuronal survival

The rats were sacrificed after anaesthetization with 200 mg/kg doses of sodium pentobarbital via intraperitoneal route. Then normal saline and subsequently paraformaldehyde (4%) in sodium phosphate buffer was perfused transcardially. The rat brains were dissected and then subjected to fixing in paraformaldehyde (4%) at a temperature of 4°C. After 3-days of fixing, the brain samples were paraffin embedded followed by slicing into 2- μ m sections. The sections were dyed by treatment with Toluidine Blue (1%) for 5 min at 60°C. The sections were examined under light microscope (model, BX53; Olympus Corporation) for calculation of viable neurons in five randomly selected fields.

Transmission electron microscopy

The brain tissues were fixed for 2 h on treatment with glutaraldehyde (2.5%) in PBS at 4°C and then with osmium tetroxide (1%; pH 7.4) for 2.5 h. The tissues were dyed using uranyl acetate (1% aqueous) solution overnight at 4°C prior to embedding in Durcupan (Sigma-Aldrich, Merck KGaA). Ultracut microtome (Leica Microsystems, Inc.) was used for cutting of hippocampus into ~ 60 nm sections which were put on formvar-coated copper grids. The sample sections were subjected to dyeing with uranyl acetate and lead citrate followed by examination under 7650 transmission electron microscopes (Hitachi High-Technologies Corporation).

Western blotting

The rats were sacrificed after anaesthetization with 200 mg/kg doses of sodium pentobarbital via intraperitoneal route. Then normal saline and subsequently paraformaldehyde (4%) in sodium phosphate buffer was perfused transcardially. The hippocampus tissues were excised and then homogenized on treatment with RIPA lysis buffer (Beyotime Institute of Biotechnology) mixed with PMSF. Centrifugation of

lysate at 4°C for 15 min at 12,000 x g was followed by protein content determination using BCA assay kit (Beyotime Institute of Biotechnology). The protein samples (30 µg) were isolated on 12% SDS-PAGE and then transferred to PVDF membranes which were blocked using SBA (3%) in TBS for 40 min at room temperature. The membrane were probed with primary antibodies at 4°C for overnight, washed with PBS and then incubated for 2 h with horseradish-peroxidase conjugated secondary antibodies (Cell Signaling Technology, Inc.). Detection and visualization of protein bands was performed using enhanced chemiluminescence system (EMD Millipore) and quantification by Quantity-One software version 4.6.3 (Bio-Rad Laboratories, Inc.). The primary antibodies used were: anti-mTOR, anti-LC3B, anti-p-mTOR, anti-Beclin-1 and anti-β-actin (Cell Signaling Technology, Inc.).

Histological analysis using Hematoxylin–Eosin (HE) staining

The tissue sections from hippocampi were perfused and subsequently fixed at 4 °C in phosphate buffer at 7.4pH. Then thin slices of hippocampi were cut using a microtome followed by rehydration in gradient sucrose solution. The sections were subsequently embedded in OCT (Tissue-Tek, Miles) and sectioned at 25 µm (Leica CM 1850, Leica Instruments). The slices after HE-staining were examined under a microscope at 200 × magnification.

Statistical analysis

The presented data are the mean ± standard deviations of three experiments. The data analysis was made by SPSS 16.0 statistical software (SPSS, Inc.). The statistical differences between groups were determined using One-Way ANOVA followed by Bonferroni tests and student's *t*-test. The differences at $P < 0.05$ were taken significant statistically.

Results

Asiaticoside improved cognitive function in VD rats

The spontaneous altered behaviors were significantly ($P < 0.05$) impaired in the VD rat model group compared to sham group (Fig. 2). Asiaticoside treatment of the VD rats significantly ($P < 0.05$) alleviated the impairment in spontaneously altered behaviors. The rapamycin administration also mediated impairment in spontaneously altered behavior in the rats. Asiaticoside failed to significantly ($P < 0.05$) alleviate spontaneously altered behavior impairment in VD rats treated with rapamycin. Asiaticoside alleviated VD mediated spontaneously altered behavior effectively when the duration between two runs was increased to 80 and 190 sec.

Asiaticoside shortened escape latency in VD rats

The escape latency showed significant increase ($P < 0.05$) in model VD group compared to rats in sham group from day 2 of dementia (Fig. 3). Treatment of VD rats with asiaticoside caused a significant ($P < 0.05$) reduction in escape latency. Rapamycin administration also increased escape latency but the

increase was not as effective as that of VD. The rapamycin mediated increase in escape latency in rats could not be reduced significantly ($P < 0.05$) by asiaticoside treatment.

Asiaticoside increased swimming time in VD rats

The distance travelled, swim time and count of platform crossings were significant ($P < 0.05$) lower for the rats in VD group (Fig. 4). However, asiaticoside significantly ($P < 0.02$) alleviated VD mediated decrease in distance travelled, swim time and count of platform crossings. Administration of rapamycin also significantly ($P < 0.05$) lowered distance travelled, swim time and count of platform crossings in the rats. However, asiaticoside treatment could not significantly ($P < 0.05$) alleviate the rapamycin mediated lowering of distance travelled, swim time and count of platform crossings.

Asiaticoside promotes neuronal survival in rats with VD

The abnormalities in hippocampus tissues of rats were detected using HE-staining (Fig. 5A) and analysis of Nissl bodies (Fig. 5B). The abnormalities were markedly evident in the hippocampus of VD rats compared to sham group. The neuronal apoptosis and degeneration was clearly seen in the hippocampus of VD rats. The VD mediated hippocampus tissue damage was alleviated significantly ($P < 0.05$) by asiaticoside treatment of the rats. The abnormalities in the hippocampus tissues were induced in the rats by rapamycin. However, no significant improvement in hippocampus tissue damage induced by rapamycin was observed on treatment with asiaticoside.

Inhibition of autophagosome formation by asiaticoside in VD rat hippocampus

The autophagosome formation was clearly detected near the nuclei in VD rat hippocampus but was absent in sham group (Fig. 6). The primary lysosome count was also increased in the VD rat hippocampus. Treatment of the VD rats with asiaticoside alleviated formation of autophagosome and markedly suppressed count of primary lysosomes. In rapamycin administered rats the autophagosome as well as lysosome count was markedly increased. No significant reduction in autophagosome formation and lysosome count was observed on rapamycin administered rats.

Asiaticoside up-regulates p-mTOR and suppresses autophagy related proteins in VD rats

The Beclin-1 and LC3II levels in VD rats were markedly elevated in hippocampus tissues compared to sham group (Fig. 7). Treatment with asiaticoside alleviated VD mediated increase in Beclin-1 and LC3II expression in the rat hippocampus tissues. The phosphorylation of mTOR in the hippocampus tissues of VD rats was suppressed markedly compared to sham control. However, asiaticoside treatment prevented suppression of mTOR phosphorylation in VD rat hippocampus tissues.

Autophagy inhibition by asiaticoside in VD rats via mTOR pathway

The rapamycin administration markedly suppressed expression of p-mTOR in rat hippocampus tissues compared to sham group (Fig. 8). On the other hand, the levels of Beclin 1 and LC3II were up-regulated markedly by rapamycin in hippocampus of rats. The rapamycin mediated suppression of p-mTOR and elevation of Beclin 1 and LC3II expression in rat hippocampus could not be alleviated by asiaticoside treatment.

Discussion

Vascular dementia one of the commonly diagnosed types of dementia is generally caused by cerebrovascular diseases and many other factors including smoking, high blood pressure and diabetes (Pulsinelli et al. 1982). The mechanism of pathology of VD is not clearly known and therefore studies are urgently required to develop effective treatments. Autophagy, the cellular adaptive process in response to stress induced by various factors in multi-cellular organisms is associated with dynamic catabolism (Levine et al. 2011). The excessive autophagy leads to death of cells and contributes significantly to neuronal damage induced by ischemia (Zhao et al. 2016; Zou et al. 2018). The studies found that neurological impairments caused by cerebral ischemia are remarkably modulated by targeting autophagy induction (Li et al. 2017; Kim and Guan 2015). The autophagy induction is regulated by mTOR kinase and it has been found that autophagy is suppressed by mTOR activation and enhanced by inhibition of Mtor (Wang et al. 2017). Autophagy is specifically induced in cells by administration of rapamycin (mTOR inhibitor) which directly inhibits mTOR (Klionsky et al. 2012). The hippocampal neuronal damage caused by hypoxia mediated injury is protected by up-regulation of mTOR pathway (Wang et al. 2012). The present study established VD rat model using the reported protocol and evaluated cognitive memory improvement by asiaticoside treatment using MWM and T-maze tests (Xing et al. 2016). The data showed 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 VD rats asiaticoside treatment prevented neuronal damage which was evident by a marked increase in Nissl-positive cell proportion compared to VD group. The neuronal damage in VD rats administered with rapamycin could not be prevented on treatment with asiaticoside. This suggests that asiaticoside prevents VD induced neuronal damage and cognitive impairment in rats but could not alleviate the effect of rapamycin.

Autophagy is associated with the transport of denatured intracellular proteins, senescent proteins and organelles with damage to lysosomes where degradation and digestion takes place. It is defense mechanism of the cells towards adverse environmental conditions encountered during various pathological processes. Studies have demonstrated that in cardiomyocytes ischemia and hypoxia induces activation of autophagy via promotion of mTOR and LC3II expression (Dai et al. 2017; Zhao et al. 2018). Cardiomyocytes are protected by appropriate level of autophagy but increased autophagy during ischemia and hypoxia results in injury to myocardial cells (Ravikumar et al. 2010). Exposure of myocardial cells to extreme level of ischemia leads to increased autophagy and promotes apoptosis during reperfusion (Yang et al. 2010). There are reports that ischemia or hypoxia induces autophagy and subsequently leads to neuronal death (Kiriya and Nochi 2015; Che et al. 2017; Jia et al. 2015; Yang et

al. 2015). The present study showed that VD induced formation of autophagosomes and enhanced the count of lysosomes in cells. The TEM examination showed absence of autophagosomes and significantly lower count of lysosomes in the VD rats treated with asiaticoside. The VD mediated autophagy activation inhibition by asiaticoside was also confirmed by western blotting. In VD rats LC3II and Beclin-1 levels were markedly higher relative to sham group. Treatment of VD rats with asiaticoside alleviated VD mediated up-regulation of LC3II and Beclin-1 expression. This data proved that asiaticoside prevents VD mediated neuronal damage by inhibition of autophagy. The effect of asiaticoside on mTOR which is a downstream executor of autophagy was also evaluated in VD rats. The present study showed that asiaticoside treatment markedly promoted expression of p-mTOR in the hippocampus tissues of VD rats. However, in VD rats administered with rapamycin asiaticoside could not inhibit induction of autophagy.

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

Declarations

Availability of data and material

The related data and material can be obtained from the authors.

Ethics approval and consent to participate

The approval for study was received Dongying District People's Hospital of Dongying City, No.333 of Jinan Road, Dongying, Shandong 257000, China.

Competing interests

The authors declare no competing interests.

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The work was self-financed.

Authors' contributions

Baohua Dong conceived and designed the study. The experiments were carried out by Min Guo, Jianmeng Xu and Shiwei Wang. Min Guo and Jianmeng Xu were also involved in data handling and processing. All the authors contributed equally in preparing the manuscript. All authors read and approved the final manuscript.

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Figures

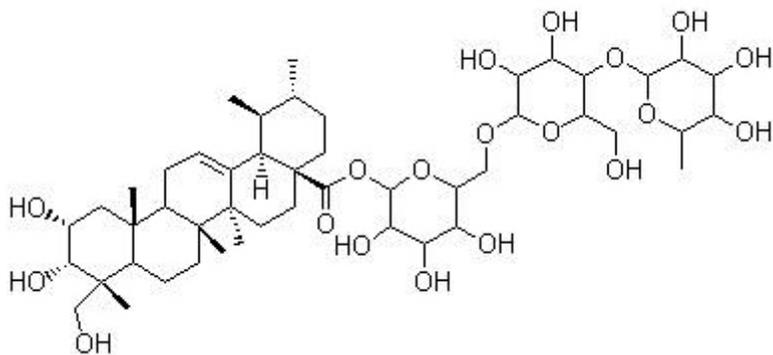


Figure 1

Chemical structure of asiaticoside.

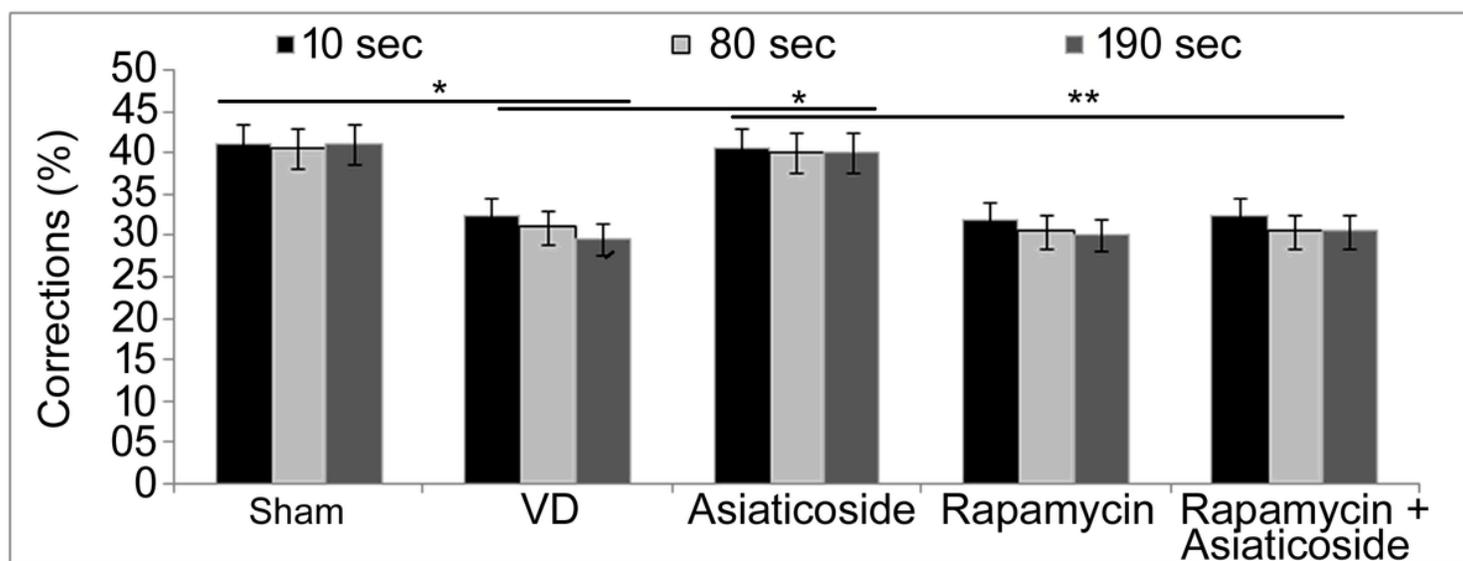


Figure 2

Effect of asiaticoside on spatial memory in VD rats. The improvement in spontaneously altered behavior impairments was analyzed in VD, sham, asiaticoside, rapamycin and asiaticoside plus rapamycin treated rats 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.

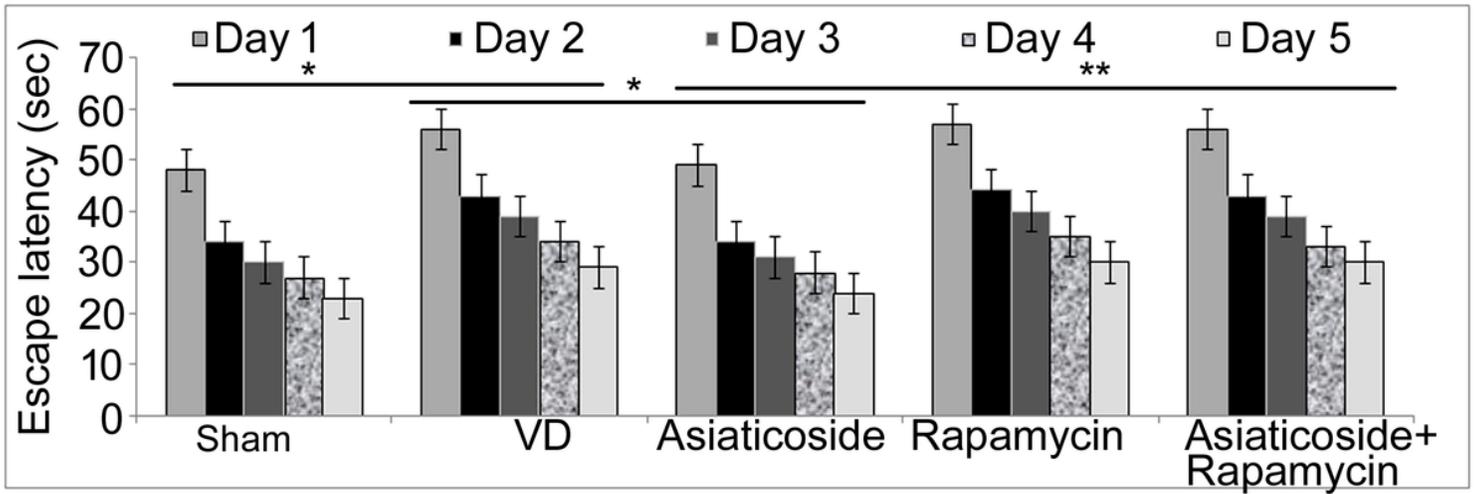


Figure 3

Effect of asiaticoside on escape latency in VD rats. The escape latencies were measured in VD, sham, asiaticoside, rapamycin and asiaticoside plus rapamycin treated rats using MWM test. The tests were conducted on day 1, 2, 3, 4 and 5 post-ischemia. * $P < 0.03$ and ** $P < 0.01$ vs. sham group.

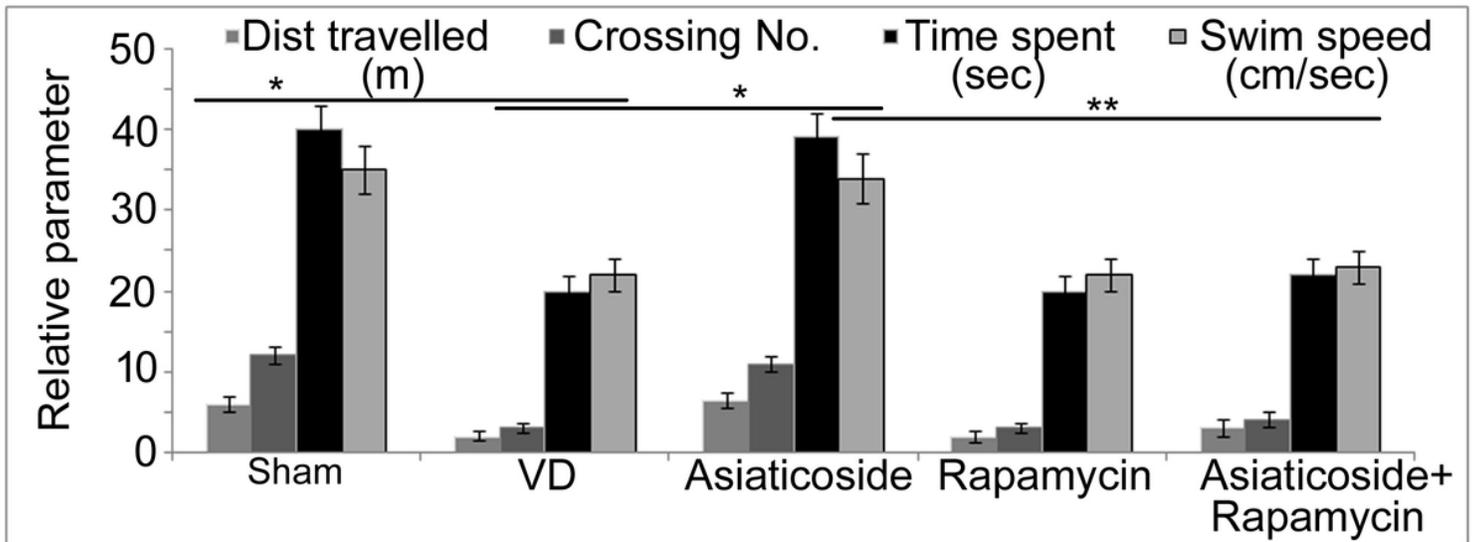


Figure 4

Effect of asiaticoside on cognition in VD rats. The distance travelled, swim time and count of platform crossings were measured in VD, sham, asiaticoside, rapamycin and asiaticoside plus rapamycin treated rats using MWM test. * $P < 0.05$ and ** $P < 0.01$ vs. sham group.

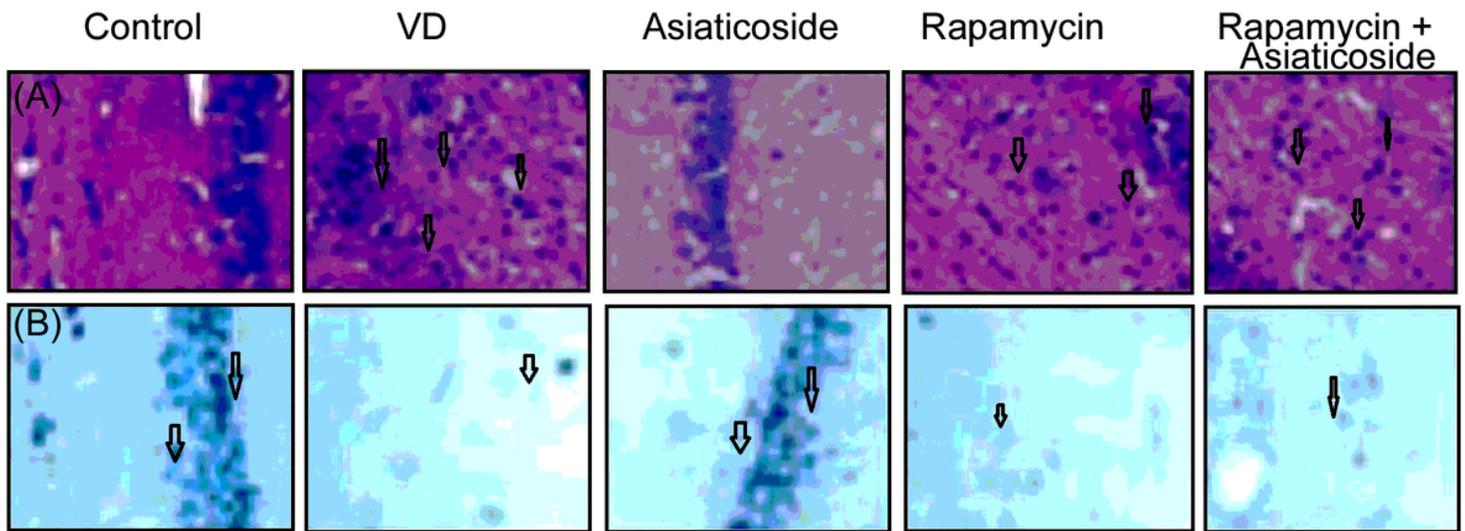


Figure 5

Effect of asiaticoside on hippocampus damage in VD rats. (A) The neuronal damage induced by VD and rapamycin in rat hippocampus following treatment with asiaticoside was detected after HE staining. (B) The Nissl bodies in rat hippocampus. Magnification x200. Arrows indicated neuronal damage.

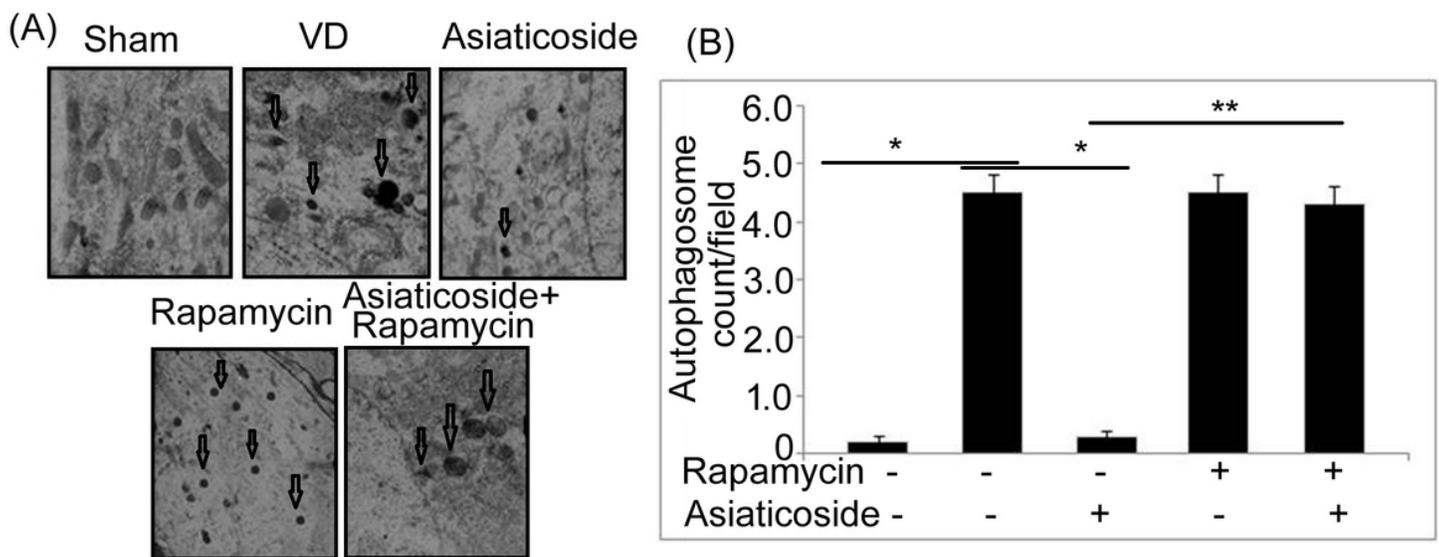


Figure 6

Effect of asiaticoside on autophagosomes in VD rat hippocampus. (A) The autophagosome formation induced by VD and rapamycin in rat hippocampus following treatment with asiaticoside was detected by electron microscopy. Magnification, x2500 (B) Quantification of autophagosome formation in rat hippocampus. * $P < 0.05$ and ** $P < 0.01$ vs. sham group. Arrows indicated autophagosomes formed.

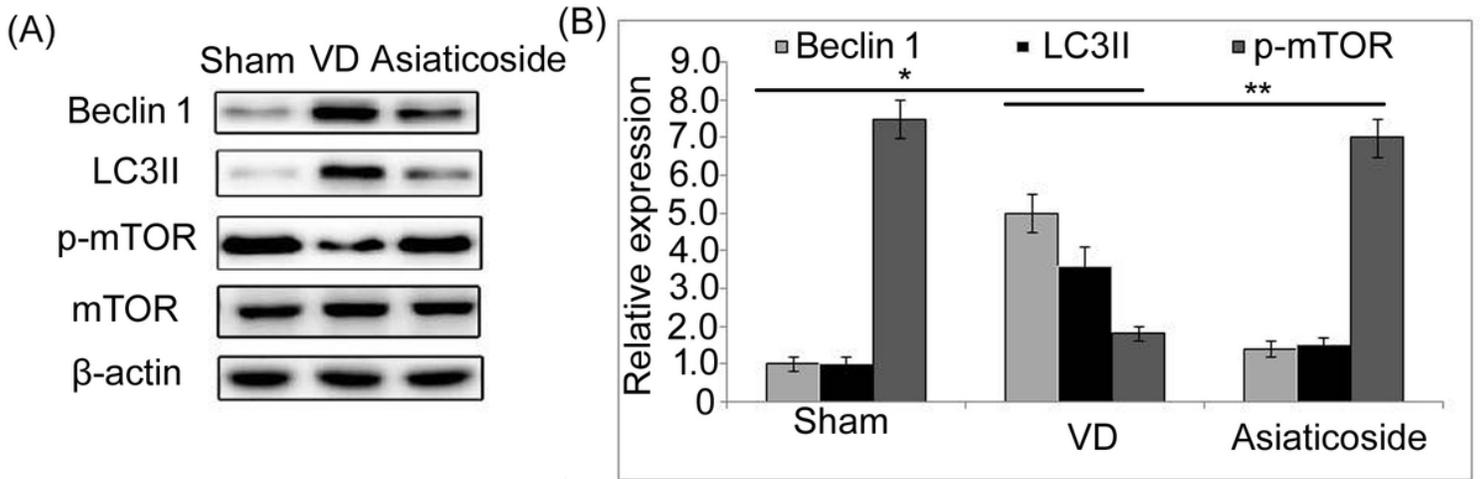


Figure 7

Effect of asiaticoside on autophagic proteins. (A) The p-mTOR, Beclin 1 and LC3II expression alteration induced by VD in rat hippocampus following treatment with asiaticoside was analyzed by western blotting. (B) Quantification of protein expression in rat hippocampus. * $P < 0.05$ and ** $P < 0.01$ vs. sham group.

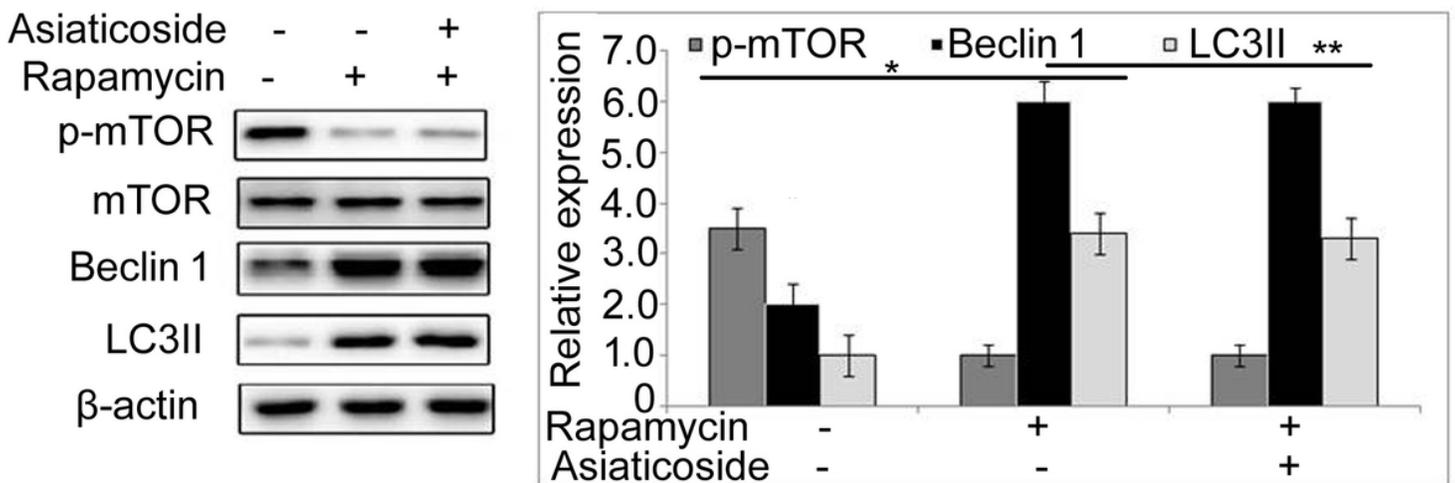


Figure 8

Effect of asiaticoside on mTOR pathway. (A) The p-mTOR, Beclin 1 and LC3II expression alteration induced by rapamycin in rat hippocampus following treatment with asiaticoside was analyzed by western blotting. (B) Quantification of protein expression in rat hippocampus. * $P < 0.05$ and ** $P < 0.01$ vs. sham group.