Asiaticoside ameliorates β-amyloid-induced learning and memory deficits in rats by inhibiting mitochondrial apoptosis and reducing inflammatory factors

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Abstract. The present study aimed to investigate the effects of asiaticoside (AS) on the pathology and associated mechanisms of β-amyloid (Aβ)-induced Alzheimer’s disease (AD) in rats. An AD rat model was established by lateral intracerebroventricular injection of Aβ 1-42 oligomers. Learning and memory function were evaluated by Morris water maze (MWM) test. In addition, hematoxylin and eosin (H&E) staining, transmission electron microscopy (TEM), immunohistochemistry, ELISA and western blot analysis were performed to evaluate the disease pathogenesis. The results indicated that AS exerted protective effects in rats treated with Aβ oligomers, in a dose-dependent manner, as evidenced by the improved learning and memory function in the MWM test. In addition, H&E staining of hippocampal tissue showed that the histological structure was damaged in the model group, which was restored by AS treatment. Aβ deposition was dramatically increased in the model group, and the pathological changes were reversed by AS treatment. TEM revealed that the subcellular structure was injured by Aβ oligomers, however, the structure was ameliorated by AS treatment. Furthermore, AS was found to reduce the elevated levels of pro-inflammatory cytokines, interleukin-6 and tumor necrosis factor-α, in the brains of Aβ-treated rats. In addition, AS treatment resulted in a significant decrease in the expression of caspases-3, whereas the expression of B-cell lymphoma-2 was significantly increased, in these Aβ-treated rats. According to the findings of the observed study, AS has a marked protective effect on Aβ-induced AD pathology, and the underlying mechanism may be associated with the alleviation of the mitochondrial injuries, the anti-inflammatory activities, and the influence on the expression levels of apoptosis-associated proteins.

Introduction

Asiaticoside (AS), a triterpenoid derivative isolated from Centella asiatica, has versatile biological effects, including antioxidant and anti-inflammatory activities, as well as protective effects against glutamate- or β-amyloid (Aβ)-induced neurotoxicity (1). In our previous study, AS has been shown to protect against apoptosis induced by Aβ 25-35 in PC12 cells, and increase the cell viability and the expression level of B-cell lymphoma 2 (Bcl-2), in a dose-dependent manner (2). Furthermore, AS has marked hepatoprotective effects on liver injury induced by lipopolysaccharide/D-galactosamine N, inhibiting the expression of tumor necrosis factor-α (TNF-α) and mitogen-activated protein kinases (3).

Alzheimer’s disease (AD) is a disorder of the neural system that is characterized by the irreversible decline of cognitive function (4). The mechanism of AD development is complex, and Aβ peptide may serve a central role in the disease pathogenesis. AD develops as a result of the overproduction and aggregation of Aβ in the brain. Aβ peptides can cause mitochondrial dysfunction and caspase activation, leading to apoptosis in neuronal cells (5). Mitochondria are metabolically active in living cells, and these organelles respond to various pathophysiological cues to provide cellular energy and meet metabolic demand (6-8). When the mitochondria are severely impaired, apoptosis and/or necrosis may occur, resulting in cell death (9-11). Concerning the effects of AS on cell viability and the role of mitochondrial-associated apoptosis in AD pathogenesis, it is of great importance to examine the effects of AS on the disease pathogenesis and the underlying mechanisms.

In the present study, the effects of AS on Aβ-induced AD pathology in rats were investigated, and the possible underlying mechanisms were also discussed. The present findings suggest the potential application of AS on AD in clinical practice in the future.

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Materials and methods

Animals and grouping. A total of 60 adult male Sprague-Dawley rats (age, 12 weeks; weight, 220-260 g) were obtained from the Laboratory Animal Centre of South West Medical University (Chongqing, China). All the experimental procedures were performed according to the ethical guidelines of Luzhou Medical College (Luzhou, China) for the care and use of laboratory animals. This study was approved by the Ethics Committee board of Luzhou Medical College. Rats were housed in an air-conditioned room at 25°C, with 55% humidity and a 12-h light/dark cycle, and fed with standard diet. They were acclimated for 5 days prior to the experiments.

The rats were randomly divided into the following groups (n=10 in each group): i) Control group, which was treated with only saline; ii) model group, in which rats were subjected to lateral intracerebroventricular infusion with Aβ 1-42 oligomers; iii) sham group, in which rats were infused with normal saline instead of Aβ 1-42 oligomers; and iv) three treatment groups, in which the rats were infused with Aβ 1-42 oligomers for 14 days, followed by the drug treatment with 5, 15 or 45 mg/kg AS.

Aβ 1-42 oligomer preparation and animal modeling. Synthetic Aβ 1-42 peptides (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in phosphate buffer solution at a final concentration of 0.45 mg/ml (or 100 µmol/l), and then stored at -80°C. The solution containing oligomers was incubated at 37°C for 7 days (12-14). Purity control and structure proof were achieved with amino acid analysis and electrospray mass spectrometry (Finnigan Mat TSQ 7000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (15).

To establish the AD model, rats were initially anesthetized with intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg body weight; Sigma-Aldrich), and then mounted on a stereotaxic apparatus (ZH-B; Shanghai Zhenghua Medical Equipment Co., Ltd., Shanghai, China). A 1.5-cm incision was made on the scalp, and the anterior fontanel was exposed. The coordinates were as follows (from the bregma): Anterior-posterior, 3.5 mm; medial-lateral, 2.0 mm; and dorsal-ventral, 2.7 mm (16). Subsequently, 10 µl Aβ 1-42 (1 µmol) was bilaterally infused with a microsyringe over a period of 10 min, and the microsyringe was kept for another 5 min before withdrawal. In the sham group, 1 µl normal saline was infused rather than Aβ 1-42, following the same procedure.

Drug administration. Asiaticoside (AS; C_21H_22O_9; molecular weight, 959.12; purity, >98%) was purchased from Guangxi Changzhou Natural Product Development Co., Ltd. (Nanning, China), and determined by high-performance liquid chromatography as previously described (1/2Q). The drug administration was performed after the AD model was established using Aβ oligomer treatment. AS was administered orally at 5, 15 or 45 mg/kg body weight per day for 7 days.

Morris water maze (MWM) test. The learning and memory function of the rats were evaluated by the MWM test 7 days after surgery, according to a previously described method (17). The swimming pool (180 cm in diameter) was maintained at 22-24°C, and divided into four quadrants with equal size. A hidden platform was placed in the center of one quadrant. Each of the cardinal points of these four quadrants was randomly selected as the start location. On the pre-test day, rats were allowed to swim freely for 120 sec. During the test (days 1-4), each rat was subjected to 8 trials each day. The trial began when a rat was placed in the pool, and ended when the rat found the platform. The escape latency was recorded. If a rat failed to find the platform within 120 sec, the trial was terminated and the escape latency was recorded as 120 sec, and the rat was guided to the platform (18,19). On day 5, the swimming path was recorded by a video recording system, and the digital images were analyzed by the water maze software (Taimeng Technology Co., Ltd., Chengdu, China). In addition, the probe test was performed, in which the platform was removed, and the rat was allowed to swim freely in the pool for 120 sec. The swimming time in the target quadrant was recorded.

Hematoxylin and eosin (H&E) staining. After the behavioral test on day 12 after surgery, the rats were deeply anesthetized with intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg body weight). The hippocampi were removed and fixed with 10% neutral-buffered formalin for 3 days, and then embedded in paraffin. Next, the samples were cut into 5-mm sections on a rotary microtome (Leitz 1512; Leica Microsystems, Wetzlar, Germany). Sections were then subjected to H&E staining according to the standard protocol, and were observed with light microscopy.

Immunohistochemistry. On day 12 after surgery, hippocampi were removed from the rats and fixed with 10% neutral-buffered formalin for 3 days, and then embedded in paraffin. The tissues were sliced at 10 µm and mounted on a slide glass. The slides were dewaxed, heated to 100°C for 30 sec in 0.01 M citric acid buffer (pH 6.0), and then cooled at room temperature. After blocking with 5% goat serum, the sections were incubated with rabbit anti-rat anti-Aβ 1-42 polyclonal antibody (1:200; 2014020; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), at 4°C overnight. Subsequent to washing with phosphate-buffered saline, the section was stained with biotin-conjugated goat anti-rabbit polyclonal IgG (1:100; 20140321; Beijing Biosynthesis Biotechnology Co., Ltd.) for 30 min, and then examined under a fluorescent microscope.

Western blot analysis. Frozen hippocampal samples were homogenated with triple detergent lysis buffer, containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mg/ml phenylmethylsulfonyl fluoride and 1 mg/ml aprotinin. Protein concentration was determined by the BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis, and then electronically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% (w/v) fat-free milk in Tris-buffered saline containing 0.05% Tween-20, followed by incubation with rabbit anti-Bcl-2 (1:5,000; ab32314; Abcam, Cambridge, MA, UK), anti-caspase-3 (1:5,000; ab13847; Abcam), or anti-β-actin (ab8227; Abcam) polyclonal antibodies. at 4°C overnight. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary
antibody (1:3,000; Cat. No., bs0295g-HRP; Beijing Biosynthesis Biotechnology Co., Ltd.). Protein bands were visualized with an enhanced chemiluminescence system (GelDocXR+; V140130; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein bands on the images and the relative protein expression were analyzed using Quantity One software (Version 4.62).

Transmission electron microscopy (TEM). The subcellular structure of the brain was detected with TEM. Briefly, rats were anesthetized and rapidly perfused with 100 ml solution containing 0.85 g sodium chloride, 0.025 g potassium chloride and 0.02 g sodium bicarbonate. Next, they were perfused with 300 ml fixing solution containing 0.5% glutaraldehyde and 4% paraformaldehyde. The entire brain was removed, fixed overnight with 0.5% glutaraldehyde and 4% paraformaldehyde, then embedded in paraffin. The sample was stained with uranium-lead (20070112; Lihua Kaishun, Beijing, China), and observed using a transmission electron microscope.

Microdialysis. Microdialysis probes (CMA 20; 0.5-mm diameter, 10-mm length, 100 kDa cutoff polyethersulfone membrane; CMA Microdialysis AB, Solna, Sweden) were injected into the brains of the rats on day 12 after surgery at 0.6 µl/min, with 0.9% (w/v) saline containing 40 mg/ml dextran (Meda AB, Solna, Sweden), as previously described (20, 21). Following a 30-min equilibration period, microdialysate was collected on ice and stored at -70°C. Next, the concentrations of interleukin-6 (IL-6) and TNF-α in the brain microdialysate were determined.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of IL-6 and TNF-α in brain microdialysate were quantified using an ELISA kit ( 20140127; R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. IL-6 and TNF-α levels were obtained according to the standard curve. All experiments were performed in triplicate.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed with SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons for multiple groups were performed using one-way analysis of variance, and comparisons between groups were performed using Student-Newman-Keuls method. P<0.05 was considered to indicate statistically significant differences among the groups.

Results

AS ameliorates the learning and memory deficit in Aβ-treated rats. To investigate the effects of AS on the learning and memory function of Aβ-treated rats, the MWM test was performed. The results demonstrated that the escape latencies of rats in the various groups did not reveal statistically significant differences on day 1 (Fig. 1A). However, on days 2-4, the escape latency of the model group was significantly greater compared with that in the control and sham groups (P<0.01; Fig. 1A). Following treatment with 15 and 45 mg/kg body weight AS, the escape latencies of the Aβ-treated rats were significantly reduced (P<0.01; Fig. 1A). Furthermore, according to the results of the swimming time in the target quadrant (Fig. 1B) and the navigation path recording (Fig. 1C), the cognitive capabilities were evidently declined in the model group compared with the control and sham groups. The cognitive capability was restored by the treatment with 5, 15 or 45 mg/kg body weight AS (P<0.01). These results suggest that AS may ameliorate the learning and memory deficit in rats with Aβ-induced AD, in a dose-dependent manner.

AS protects hippocampi against the impairment caused by Aβ oligomers. To investigate the effects of Aβ oligomers and
AS on hippocampi in rats, H&E staining was performed. The results indicated that, in the control and sham groups, complete hippocampal neurons and cells in surrounding tissues were observed, with integrated cell membrane, uniform-colored cytoplasm and round nuclei. However, in the model group, the structure of hippocampal neurons was incomplete, the connection with cells in the surrounding tissues was weak, and edema and bubbles were observed, indicating cell death. By contrast, treatment with 5, 15 and 45 mg/kg body weight AS significantly ameliorated the impairments in the hippocampal region caused by Aβ oligomers (Fig. 2), in a dose-dependent manner. These results suggest that AS treatment protected the hippocampi from Aβ-induced impairment.

AS decreases Aβ deposits in the hippocampus of rats treated with Aβ oligomers. To further investigate the effects of AS treatment on Aβ-induced pathology in rats, immunohistochemistry was performed. The results demonstrated that, compared with the control and sham groups, the Aβ deposits were significantly increased in the model group (Fig. 3). However, when treated with AS, the numbers of Aβ deposits in the hippocampus of these model rats was significantly deceased (Fig. 3). These results suggest that AS reversed the pathological changes in the hippocampal region caused by Aβ oligomers.

AS ameliorates impaired subcellular structure in rats treated with Aβ oligomers. To investigate the effects of Aβ oligomers and AS treatment on the subcellular structure in rat brains, TEM was performed. The TEM results indicated that the nuclear structure in hippocampal neurons was intact in the control and sham groups, with a clear double membrane, complete outer and inner membranes of mitochondria and intact cristae observed. However, in the model group, the hippocampal neurons were evidently damaged, exhibiting abnormal nuclear membrane and swelling mitochondria with vague cristae (Fig. 4). The treatment with AS significantly ameliorated the pathological changes in the subcellular structure of hippocampal neurons that was induced by Aβ oligomers. The nuclear membrane was clear and intact, while the outer and inner membranes of mitochondria were complete, with normal cristae. These results suggest that AS treatment ameliorated the impairments in the subcellular structure in the hippocampal region of Aβ-treated rats.

AS treatment decreases caspase-3 and increases Bcl-2 expression in Aβ-treated rats. To investigate the effects of Aβ oligomers and AS treatment on apoptosis-associated proteins, the expression levels of caspase-3 and Bcl-2 in the hippocampus of rats were detected by western blot analysis. The results showed that the expression of caspase-3 was increased and the expression of Bcl-2 was decreased in the model group, when compared with that in the control and sham groups (P<0.01; Fig. 6). However, when treated with AS, the expression of caspases-3 was significantly decreased, and the expression of Bcl-2 was significantly increased in the AD model rats.
These results suggest that AS treatment altered the expression levels of apoptosis-associated proteins in rats treated with Aβ oligomers, which may contribute to its protective effects in the disease pathogenesis.

**Discussion**

AD is a progressive neurodegenerative disorder, clinically characterized by the impairment of cognitive function and the changes in behavior and personality (22,23). One hallmark of AD is the amyloid deposition, the major component of which is Aβ peptide with 40–42 residues (24). The pathogenic factors for AD include oxidative stress, reactive oxygen species cytotoxicity, mitochondrial dysfunction and apoptosis, and inflammatory responses (25-27). Excessive accumulation of Aβ oligomers in the brain serves an important role in the disease pathogenesis, particularly concerning cognitive deficits.
Previous studies have indicated that intracerebroventricular injection of Aβ1-42 oligomers at a nanomolar dose can dramatically impair the learning and memory function in rats (28), accompanied by increased apoptosis in the hippocampus and high levels of pro-inflammatory cytokines in the brain (29,30). Furthermore, intracerebroventricular injection of low doses of Aβ25-35 (31) or Aβ1-28 (32,33) has also been shown to influence the spatial memory function and Y-maze alternation behavior in mice. MWM spatial learning test is typically used in the evaluation of rodent models for cognitive disorders and possible treatments (34,35). In the present study, Aβ1-42 oligomers were administrated to establish a AD rat model, and the spatial learning and memory function of rats was assessed by the MWM test. The effects of AS administration on the disease pathogenesis and the associated mechanisms of its effect were also investigated. The results revealed that, on days 4-5, cognitive and memory functionality reduction was observed in the rats treated with Aβ oligomers.

Centella asiatica (L.) Urban (Umbelliferae), which is widely distributed in south China, has been used as a folk medicine agent for the improvement of learning and memory ability, and for treating neurological disorders (1,36). AS is one of the active ingredients of Centella asiatica, responsible for its pharmacological activity. AS exerts various therapeutic effects, such as heat removal, diuresis promotion, detumescence and detoxification, and has been used in the treatment of urinary tract infections, skin diseases, jaundice, diarrhea, measles and larynx gall injuries (37). Although a recent study has demonstrated that AS also exerts nerve protective effects (38), its role on AD pathogenesis and the associate mechanisms of its action, particularly concerning the pro-inflammatory cytokine levels and subcellular structure, have not been fully elucidated (39). The results of the present study revealed that
the escape latency time was significantly longer in the model group compared with that in the AS-treated group. In the probe test, the time the rats in the model group spent in the target quadrant and the swimming distance recorded were significantly reduced, when compared with those in the AS treatment groups. These findings suggested that AS was able to ameliorate the declined cognitive function in rats treated with Aβ oligomers, and attenuated the neurotoxicity induced by the peptides.

The mitochondrial cascade hypothesis claims that mitochondrial dysfunction is the primary and leading event in AD pathology (7). Evidence shows that the progressive accumulation of Aβ peptides in mitochondria may induce mitochondrial-mediated toxicity (40). Along with energy metabolism, mitochondria serve a pivotal role in the survival and death of neurons through the regulation of the apoptotic pathways, by releasing cytochrome c and other pro-apoptotic factors (41). In the present study, TEM analysis demonstrated that the structure of nuclei and mitochondria in the hippocampus of the control and sham groups was intact, with a complete membrane observed; by contrast, in the model group, the subcellular structure was evidently damaged. Following treatment with AS, the injuries in the mitochondria in hippocampal neurons was restored in these model rats.

To investigate the possible mechanisms underlying the protective effects of AS against AD, the levels of pro-inflammatory factors IL-6 and TNF-α in the rat brains were measured with microdialysis probes. Pro-inflammatory cytokines serve important roles in AD pathogenesis, and the alterations in their serum levels have been observed in AD patients (42,43). The inflammatory response associated with the activation of microglia and astrocytes is an important factor in AD pathology, as evidenced by post-mortem analysis of AD brains and studies in animal models (44-46). Inflammatory response is presumably triggered by soluble Aβ peptides or fibrils, leading to microglial activation, particularly in the vicinity of neuritic plaques. These activated microglia exhibit altered morphology, and produce interleukins, interferons, chemokines and components of the complement system (47,48). Pro-inflammatory cytokines released by activated microglia, including IL-1β, IL-6, IL-8 and TNF-α, have been implicated in neurodegeneration (49). In the present study, the contents of IL-6 and TNF-α in the brain dialysate were found to be markedly increased in the model group, when compared with the control and sham groups, while treatment with AS decreased the levels of these pro-inflammatory cytokines.

Numerous studies have demonstrated that Aβ is a predominant factor in the pathogenesis of AD, and that cellular apoptosis is involved in the disease pathology (50). Caspase-3 and Bcl-2 are important participants in the process of apoptosis, and their expression levels can determine the cell survival and death (51). Activated caspase-3 has been reported to be present in AD brains (44,52) and amyloid precursor protein transgenic mice (53,54). Notably, Aβ 1-42 has been shown to induce cytochrome c release from mitochondria (55), which can activate caspase-3 and initiate apoptosis. By contrast, Bcl-2 is an apoptosis-inhibiting protein, which prevents cellular apoptosis. In the current study, the expression levels of caspase-3 and Bcl-2 were also detected. The current study results demonstrated that AS treatment decreased the expression of caspase-3 and increased the expression of Bcl-2 in the AD model rats.

In conclusion, the results of the present study revealed that AS exerted neuroprotective effects in an AD rat model induced by intracerebroventricular injection of Aβ 1-42 oligomers, in a dose-dependent manner. AS alleviated the impairment in learning and memory function, decreased the Aβ deposition in the hippocampus and restored the damage in the subcellular structure. Furthermore, the results showed that AS reduced the pro-inflammatory factor levels of IL-6 and TNF-α, decreased the expression levels of caspase-3 and increased the expression levels of Bcl-2. These results suggest that AS has a notable protective effects on Aβ-induced AD pathology, and the mechanism underlying its action may be associated with the alleviation of the mitochondrial injuries, its anti-inflammatory activities and its effect on the expression levels of apoptosis-associated proteins.

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