

Research Article

Protective role of Dihydromyricetin in Alzheimer's disease rat model associated with activating AMPK/SIRT1 signaling pathway

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The aim of the present study was to understand the possible role of the Dihydromyricetin (DHM) in Alzheimer's disease (AD) rat model through regulation of the AMPK/SIRT1 signaling pathway. Rats were divided into Sham group, AD group, AD + DHM (100 mg/kg) group and AD + DHM (200 mg/kg) group. The spatial learning and memory abilities of rats were assessed by Morris Water Maze. Then, the inflammatory cytokines expressions were determined by radioimmunoassay while expressions of AMPK/SIRT1 pathway-related proteins by Western blot; and the apoptosis of hippocampal cells was detected by TdT-mediated dUTP nick end labeling assay. AD rats had an extended escape latency with decreases in the number of platform crossings, the target quadrant residence time, as well as swimming speed, and the inflammatory cytokines in serum and hippocampus were significantly elevated but AMPK/SIRT1 pathway-related proteins were reduced. Meanwhile, the apoptosis of hippocampal cells was significantly up-regulated with decreased Bcl-2 and increased Bax, as compared with Sham rats (all $P < 0.05$). After AD rats treated with 100 or 200 mg/kg of DHM, the above effects were significantly reversed, resulting in a completely opposite tendency, and especially with 200 mg/kg DHM treatment, the improvement of AD rats was more obvious. DHM exerts protective role in AD via up-regulation of AMPK/SIRT1 pathway to inhibit inflammatory responses and hippocampal cell apoptosis and ameliorate cognitive function.

Introduction

As a major type of dementia in aging population, Alzheimer's disease (AD) has been widely deemed as a multifactorial degenerative disease in central nerve system with the characteristics of progressive cognitive decline or dysfunction [1,2]. At present, AD, only secondary to the cardiovascular disease or tumors, has risen to the third place among all diseases, costly and severely distressing for patients and their families [3–5]. AD, as the increasing epidemic threatening public health, is quite complicated mainly involving two neuropathological hallmarks: senile plaques and neurofibrillary tangles, which consisted of amyloid- β ($A\beta$) peptides and hyperphosphorylated tau protein, respectively [6,7]. As reported, the deposition and aggregation of the $A\beta$ peptide have been shown on numerous occasions, which could trigger inflammatory responses and cause neuronal dysfunction, and ultimately resulting in dementia or AD [8,9]. Although much progress has been made in the AD treatment in recent years, the efficacy is not very satisfactory. Thus, understanding the molecular mechanism of AD is of great significance for AD treatment [10].

Dihydromyricetin (DHM), also known as ampelopsin (AMP), is a kind of natural flavonoids compounds and the major functional ingredient of *Ampelopsis grossedentata*, with physiological functions

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like anti-inflammation, anti-oxidation and enhancement in immunity [11]. As demonstrated previously, DHM can activate the AMPK signaling pathway to induce the autophagy, and thereby improving skeletal muscle insulin resistance and hyperglycemia-induced cell injuries [12,13]. Moreover, Kou et al. [14] pointed out that DHM can also alleviate the skeletal muscle atrophy in aging rats through activation of AMPK/SIRT1/PGC-1 α signaling pathway. As for AMP-activated protein kinase (AMPK), it is a member of serine/threonine protein kinase extensively existing in eukaryotic cells, which could be acted as an important energy sensor to maintain the cellular energy balance [15]. Recently, AMPK was suggested to play a role in controlling the aging process [16]. In terms of SIRT1, a NAD⁺-dependent histone deacetylase, it is a major downstream molecule in AMPK signaling pathway, with a close correlation to the pathogenesis of aging-related diseases, like neurodegenerative diseases [17]. Of note, Shah et al. [18] revealed the involvement of AMPK/SIRT1 signaling pathway in the modulation of A β deposition and cognitive functions in AD rats. As such, we speculated that the potential regulatory role of DHM in the AMPK/SIRT1 signaling pathway might be crucial to the aging-related diseases, like AD.

Therefore, using the AD model of rats, the present study was done to verify whether DHM can exert the regulatory roles in AD through mediating the AMPK/SIRT1 signaling pathway, in order to provide potential strategy for the clinical treatment of AD.

Materials and methods

Ethics statement

All animal work described in the present study was approved by the Ethic Committee of Qingdao Mental Health Center. The protocols regarding the animal experiments were stipulated and implemented in strictly accordance with the Guide for the Care and Use of Laboratory Animals stipulated by National Institute of Health (NIH) [19].

Model construction and grouping

A total of 40 pathogen-free Sprague Dawley (SD) male rats (age: 8 weeks; bodyweight: 250–300 g) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. and raised in the laboratory for 1 week of adaption in a 12/12 light/dark cycle with the temperature of $21 \pm 2^\circ\text{C}$ and free access to water and food. Then, rats were randomly divided into Sham group, AD group, AD + DHM (100 mg/kg) group and AD + DHM (200 mg/kg) group, with 10 rats per each group. AD models were established as follows: rats were anesthetized of 10% chloral hydrate (300 mg/kg) by intraperitoneal injection and fixed in the stereotaxic apparatus in prone position. Then, the intrahippocampal injection of amyloid- β_{1-42} (A β_{1-42}) oligomers (5 μl , Sigma, St. Louis, MO, U.S.A.) was performed bilaterally using the micro-syringe with coordinates (from bregma): anteroposterior, 3 mm; mediolateral, 2 mm; dorsoventral, 3.5 mm [20]. The validity of AD model was indicated by severe cognitive deficit, impaired hippocampal synaptic plasticity and marked increase levels of β -site amyloid precursor protein (APP) cleaving enzyme [21], and all rat injected with A β_{1-42} were confirmed to be successfully established of the model. While 5 μl of 0.9% normal saline instead of A β solution was injected in rats from the Sham group. Two hours prior to the A β infusions, rats in the AD + DHM (100 mg/kg) group and AD + DHM (200 mg/kg) group were additionally received the intraperitoneal injection of DHM in doses of 100 and 200 mg/kg, respectively. The dosage of the DHM was chosen according to a previous study [14]. Drug administration lasted for 21 days. DHM (CAS No. 27200-12-0) purified by high-performance liquid chromatography with the purity of more than 98% was purchased from Zelang Medical Technological Co. Ltd. (Nanjing, China). Chemical structure and chromatograms of DHM were shown in Supplementary Figure S1.

Morris water maze

Place navigation test was performed immediately after the drug administration. The MWM task lasted for 5 days. The first day represented the place navigation test. In brief, rats were placed in the water with their head facing the wall, randomly from any of four quadrants, and the time(s) of rats searching for the platform was recorded. Any rats failed in arriving at the platform in 120 s were guided to the platform, allowing them to stay on the platform for 30 s, and their stay in water was recorded as 120 s. After test, rats were dried before being placed into the cages. After consecutive 5 days of training (twice per day), we recorded the time(s) of rats in searching for the platform in each quadrant, with the average of escape latencies in four quadrants as the final latency of this day. Spatial probe test was carried out at the sixth day with no platform in the maze, and rats were placed in a labeled place in the quadrant. In this test, we recorded the times that rats passed through the position of platform. This test was performed twice a day (morning and afternoon), and the average was used as the final result, and the swimming speed was recorded.

Sample preparation

After MWM, five rats in each group were anesthetized by intraperitoneal injection of chloral hydrate. Through the incision in the chest, the heart was exposed, where a small incision was made at the apex for inserting a perfusion needle. Right auricle was removed, and 150 ml of 0.9% normal saline was perfused rapidly, followed by perfusion with 300 ml of 4% neutral paraformaldehyde (PFA). Brain tissues were taken out and placed in 4% neutral PFA. The remaining five rats were decapitated under anesthesia to collect the brain tissues, from which the hippocampus was taken out rapidly on ice and preserved at -80°C for further use.

Radioimmunoassay

Under anesthesia, blood samples were collected from the heart, and after centrifugation, supernatant was transferred in new Eppendorf (EP) tubes for further use. Meanwhile, homogenate of hippocampus was also centrifuged to obtain the supernatant. Interleukin (IL)- 1β , tumor necrosis factor (TNF)- α and IL-6 levels were detected in the serum and hippocampus tissues of rats in strictly accordance with the instructions of radioimmunoassay kit.

Hematoxylin & eosin and Congo Red staining

Brain tissues fixed in 4% neutral PFA were embedded in paraffin and sliced serially into 4- μm -thickness sections. Following dewaxing in xylene and hydrating with alcohol in gradient concentrations, sections were rinsed using tap water and stained with hematoxylin (Biohao Biotechnology Institute, Wuhan, China) for 1 min. Residual hematoxylin was rinsed using tap water. Sections were differentiated using 1% hydrochloric acid-ethanol, blued in 1% ammonia hydroxide for 30 s, stained with eosin for 2 min, dehydrated with alcohol in gradient concentrations, fixed in xylene and sealed with neutral balsam. The morphological changes were observed under a microscope. The experiment was repeated three times. For Congo Red staining, deparaffinized and rehydrated sections were first stained in Gill's hematoxylin solution (Sigma, St. Louis) for 10 min and then rinsed in running tap water for 5 min and incubated in alkaline sodium chloride solution for 20 min. Sections were then stained in Congo Red working solution (Sigma) for 15 min, followed by dehydration through 95% alcohol. They were then dehydrated, hyalinized and mounted for microscopic examination.

TUNEL methods

Brain sections were fixed in 4% neutral PFA and taken out for paraffin embedding. Then, sections were dewaxed in xylene and hydrated in ethanol in gradient concentrations. Following rinsing sections in tap water, antigen retrieval was implemented at 80°C . Thereafter, the sections were incubated in presence of Protease K (Sigma, U.S.A.) and then placed in TDT buffer (Sigma, U.S.A.) for pre-incubation. After washed with PBS again, the sections were then incubated with anti-digoxin and anti-serum alkaline phosphatase complex (Sigma, U.S.A.) at 37°C overnight. Following washes in Tris-buffer, counter staining was performed with the corresponding reagent, and ended using Tris-buffer. Apoptosis in hippocampus of rats was observed under a microscope, and the results were quantified in Image-Pro Plus. Apoptotic ratio is calculated using the formula: Apoptotic ratio = TUNEL-positive cell quantity/total quantity of cells. The experiment was repeated three times.

Western blot assay

Following snap freezing in liquid nitrogen, hippocampus lysate was prepared using radioimmunoassay reagent for 30 min and centrifuged at 4°C for 30 min at 12000 g. Protein concentration was measured using the bicinchoninic acid (BCA) kit (Beyotime, Beijing, China). Protein extracts, together with loading buffer, were heated at 95°C for 10 min, and according to the relative molecular weight of targeted proteins, 6–12% separation gel was prepared (Boster, Wuhan, China) for separation of proteins. Then, proteins on the gel were transferred onto the polyvinylidene fluoride (PVDF) membrane (Millipore, U.S.A.) that were then blocked with 5% bovine serum albumin for 1 h at room temperature. Membranes were then incubated with the primary antibodies, such as anti-AMPK, anti-p-AMPK, anti-SIRT1, anti-Bcl-2, anti-Bax, anti-NF- κB p65 and anti-GAPDH (Cell Signaling Technologies, U.S.A.) at 4°C overnight. After washed with Tris-buffer saline with Tween 20 for three times (5 min/time), blots were then probed with the corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. The target protein was visualized by the enhanced chemiluminescence (ECL) reagent (Beyotime, Beijing, China). Targeted blots were quantified by ImageJ software with GAPDH as internal control. The experiment was repeated three times.

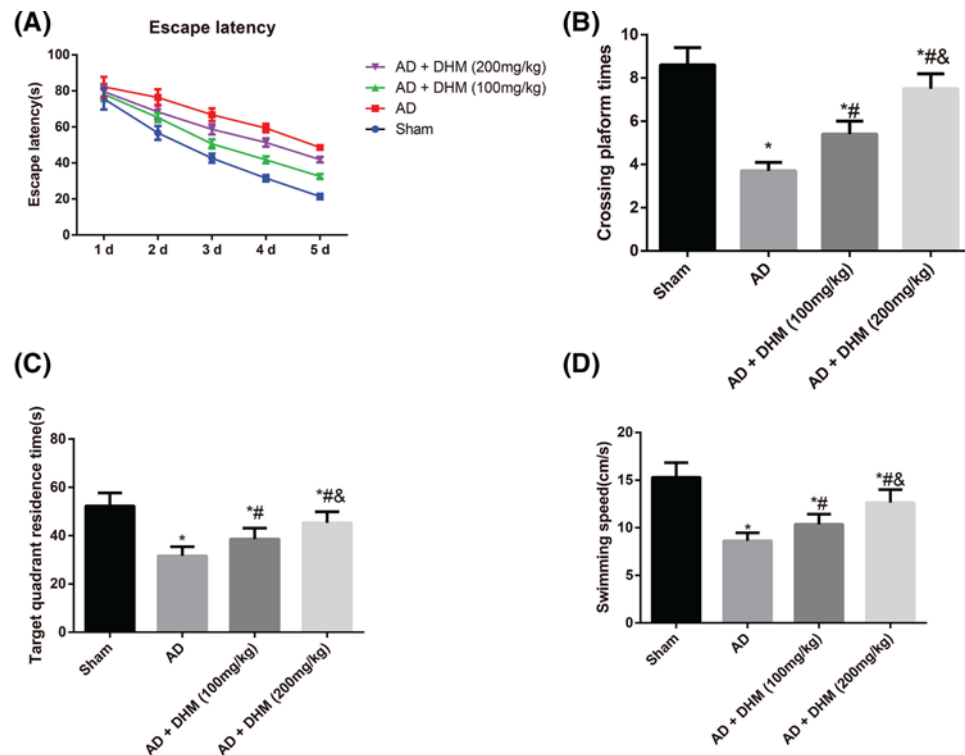


Figure 1. Examination of the spatial learning and memory abilities of rats by MWM

Comparison of the escape latency of rats (A), the numbers of platform crossings (B), target quadrant residence time (C) and the swimming speed (D) in different groups. Data were indicated as mean \pm standard deviation values ($n=10$). * $P<0.05$ vs. the Sham group; # $P<0.05$ vs. the AD group; & $P<0.05$ vs. the AD + DHM (100 mg/kg) group.

Statistical methods

All data were analyzed using SPSS 21.0 software (SPSS Inc, Chicago, IL, U.S.A.). Measurement data, in form of mean \pm standard deviation ($\bar{x} \pm s$), were compared by t -test between groups. One-way ANOVA test was used to compare differences among multiple groups followed by Tukey's HSD Post Hoc test to compare significance between groups. $P<0.05$ considered as being statistically significant.

Results

Comparison of the spatial learning and memory abilities of rats in different groups

As shown in Figure 1, the AD rats had evident decreases in spatial exploration ability and learning and memory abilities, as evident by the prolonged escape latency, reduced numbers of platform crossings, shortened target quadrant residence time and decreased swimming speed, when compared with that in the Sham group (all $P<0.05$). However, after 100 or 200 mg/kg of DHM treatment in AD rats, the shortened escape latency, increased numbers of platform crossings, prolonged target quadrant residence time, as well as increased swimming speed, were observed ($P<0.05$), and these changes was more obvious in AD rats treated with 200 mg/kg of DHM ($P<0.05$).

Effects of DHM on the morphological changes of rats in different groups

Morphological changes in the hippocampus of rats were observed by using hematoxylin & eosin (HE) staining, and the results are listed in Figure 2A. In the Sham group, the hippocampal neurons of rats were lightly stained for intracellular structures and nucleus. In contrast, the layers and numbers of hippocampal cells of the AD rats were decreased with enlarged intercellular space and disordered cells; especially, some cells exhibited shrink in volume, with pyknosis or rupture in nuclei, and cells were deeply stained into red. After AD rats treated with 100–200 mg/kg DHM, the hippocampal neurons were evenly stained and arranged closely, and the neuron cells were increased, which was more

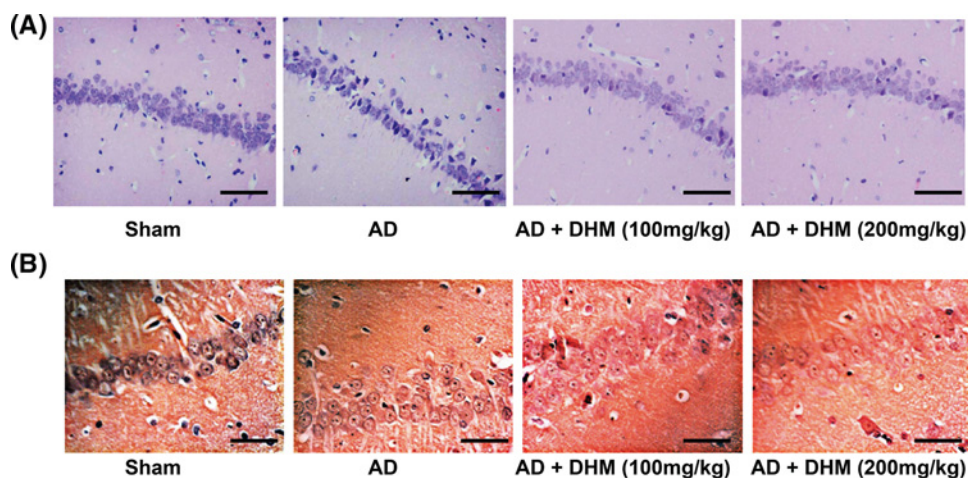


Figure 2. The morphological changes of the hippocampus of rats by HE(A) and congo red staining (B) ($\times 400$, $n=5$)
Scale bar = 50 μm .

significant amelioration in those treated with 200 mg/kg of DHM. Congo Red staining (Figure 2B) was used to determine the amyloid plaques deposition in the hippocampus. Amyloid plaques exhibited a light red dispersion without distinct boundaries and the amount of amyloid deposition in the AD rats appeared to be increased as compared with the hippocampi of the Sham group. Following DHM treatment at two different doses, the cells were stained lighter with clear cellular structure as compared with the rats in the AD group.

Comparison of the inflammatory cytokines in serum and hippocampus of rats in different groups

Compared with the Sham group, rats in the AD group had significantly increased in the levels of IL-1 β , IL-6 and TNF- α in serum and hippocampus (all $P < 0.05$, Figure 3). Nevertheless, AD rats treatment with DHM (100 mg/kg) group and DHM (200 mg/kg) significantly reduced the serum and hippocampus levels of IL-1 β , IL-6 and TNF- α in rats, and the reductions in the AD + DHM (200 mg/kg) group were much more evident than those in the former group (all $P < 0.05$).

Comparison of the hippocampal neuronal apoptosis of rats in different groups

TdT-mediated dUTP nick end labeling (TUNEL) staining method was applied to determinate the hippocampal neuronal apoptosis of rats in different groups (Figure 4). In comparison with the Sham group, rats in the AD group had a significant increase in the apoptotic ratio of the hippocampus ($P < 0.05$). When compared with the AD group, the apoptotic ratios of the hippocampus in rats from the AD + DHM (100 mg/kg) group and AD + DHM (200 mg/kg) group were significantly decreased, and especially lower in AD rats with 200 mg/kg of DHM treatment (all $P < 0.05$).

Expressions of AMPK/SIRT1 signaling pathway-related protein in rats

As shown in Figure 5, the protein expressions of p-AMPK/AMPK and SIRT1 in the hippocampus of rats from the AD group exhibited a significant decline, with an evident reduction of Bcl-2 and elevations of Bax and NF- κB p65 (all $P < 0.05$). These changes were rescued after administration of either 100 or 200 mg/kg of DHM, which had more evident alterations in rats with 200 mg/kg of DHM.

Discussion

Intracerebroventricular injection of A β_{1-42} has been shown to induce the behavioral and pathological characteristics of AD, like learning deficits and hippocampal damage, which has been used as a common method for the preparation of AD models [22,23]. A β_{1-42} , developed from the partial hydrolysis of APP, has been thought to be a critical step in AD pathogenesis, since its accumulation led to amyloid fibril formation and the generation of senile plaques [24,25]. Therefore, in the present study, the AD rat models induced by A β_{1-42} in our study had the prolonged escape latency with decreases in the number of platform crossings and the percentage time spent in the target quadrant, which was

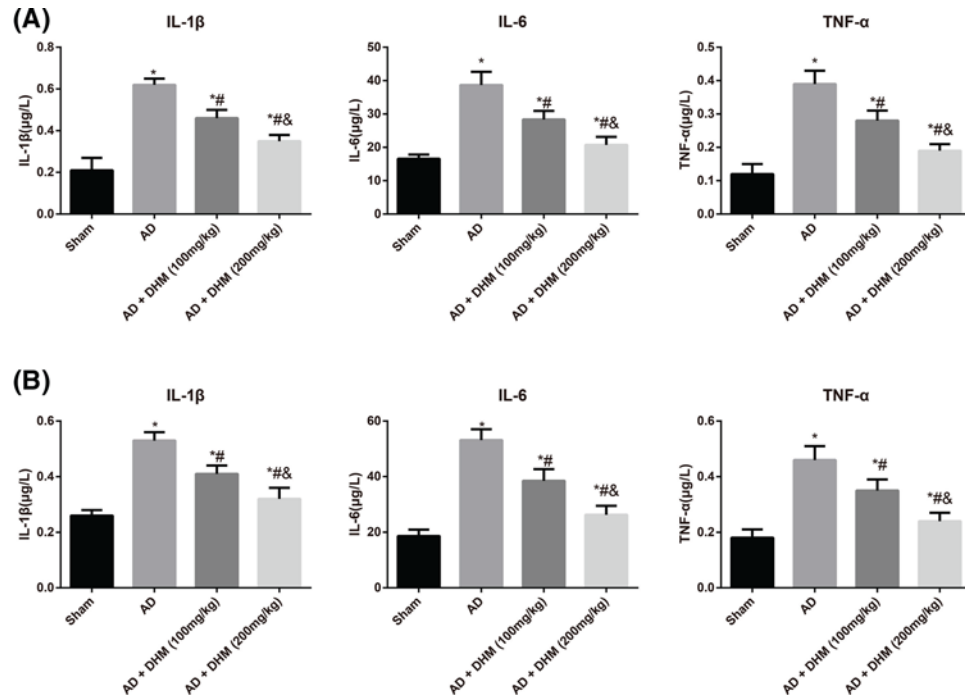


Figure 3. Comparison of the inflammatory cytokines in serum and hippocampus of rats in different groups
 Data were indicated as mean ± standard deviation values (n=5); *P<0.05 vs. the Sham group; #P<0.05 vs. the AD group; &P<0.05 vs. the AD + DHM (100 mg/kg) group.

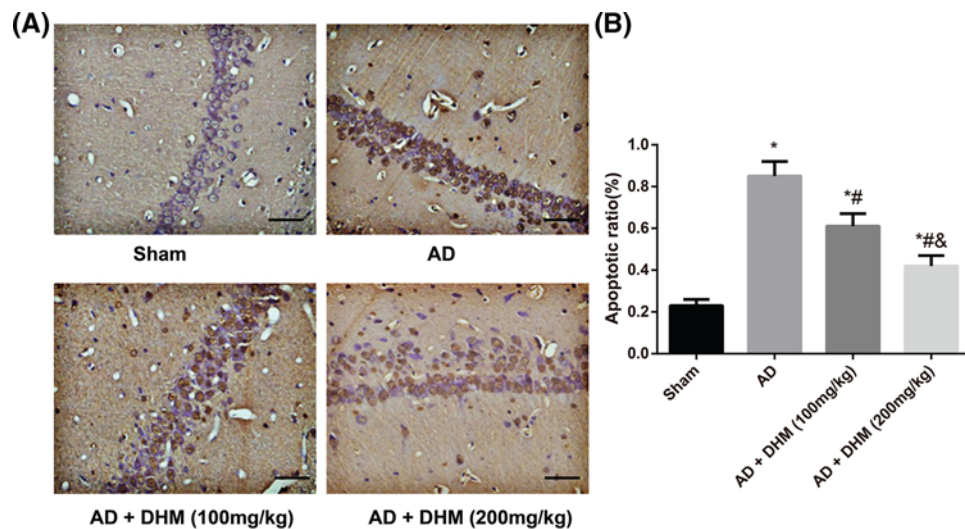


Figure 4. Detection of the hippocampal neuronal apoptosis of rats in different groups by TUNEL staining (×400); scale bar = 50 µm

(A) Detection of the hippocampal neuronal apoptosis of rats in different groups using TUNEL staining. (B) Comparison of the apoptotic ratio of rats in different groups. Data were indicated as mean ± standard deviation values (n=5); *P<0.05 vs. the Sham group; #P<0.05 vs. the AD group; &P<0.05 vs. the AD + DHM (100 mg/kg) group.

consistent with the previous observations [26,27], exhibiting an evident cognitive impairment in AD rats. Meanwhile, HE staining also revealed the pathological alterations of hippocampal cells, which confirmed the successful establishment of AD models through the behavioral and pathological perspectives. However, after treated with DHM, either 100 or 200 mg/kg (these concentrations have been confirmed to be safety), the learning and memory abilities of AD rats were elevated with significant alleviations in the morphological and pathological changes of hippocampus,

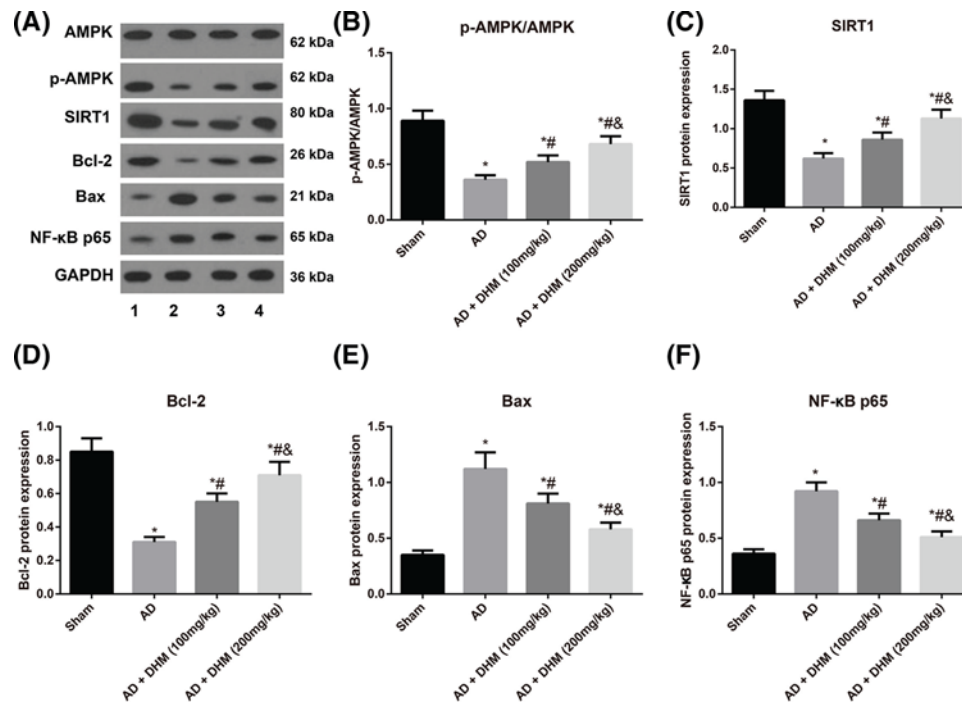


Figure 5. Expressions of AMPK/SIRT1 signaling pathway-related protein in rats detected by Western blot

(A) Expressions of AMPK/SIRT1 signaling pathway-related protein in rats detected by Western blot, 1, Sham group, 2, AD group, 3, AD + DHM (100 mg/kg) group, 4, AD + DHM (200 mg/kg) group. (B–F) Comparisons of the protein expressions of p-AMPK/AMPK, SIRT1, Bcl-2, Bax and NF-κB p65 of rats in different groups. * $P < 0.05$ vs. the Sham group; # $P < 0.05$ vs. the AD group; & $P < 0.05$ vs. the AD + DHM (100 mg/kg) group.

suggesting that DHM may protect rats from the $A\beta_{1-42}$ -induced cognitive dysfunction. Furthermore, the serum and hippocampus levels of inflammatory cytokines in rats were then determined by radioimmunoassay, and consequently, the significant increased levels of IL-1 β , IL-6 and TNF- α were observed in the serum and hippocampus of AD rats. Worth mentioning, AD-related inflammatory reaction was partially associated with the activation of microglial cells and astrocytes surrounding the $A\beta$ deposits [28,29]. Moreover, microglial cells, a kind of immunocytes, could secrete the inflammatory cytokines, including IL-6 and TNF- α , when activated; while the activation of astrocytes also triggered the massive generation of inflammatory mediators, like IL-1 β , IL-6 and TNF- α etc. [30]. And these inflammatory cytokines usually resulted in neuronal necrosis and lesions, giving rise to cognitive dysfunction [31]. Importantly, the serum and hippocampus levels of inflammatory cytokines were obviously decreased in rats with DHM treatment. Similarly, DHM can also alleviate the LPS-induced neuroinflammation in microglial cells through suppression of nuclear factor- κ B (NF- κ B) and JAK2/STAT3 pathway, as indicated by Weng et al. [32], showing that DHM may have an anti-inflammatory effect on $A\beta_{1-42}$ -induced neuroinflammatory responses.

In addition, inhibition of AMPK/SIRT1 signaling pathway was further discovered in AD rats but was reversed after different doses of DHM treatments. In the study of Kou et al., DHM can also activate the AMPK/SIRT1/PGC-1 α signaling pathway in D-gal-induced aging rats [14]. There was evidence believed that the modulation effect of AMPK/SIRT1 pathway was mainly achieved by the down-regulation of transcript factors (NF- κ B and AP-1), as well as the acetylation of histone [33]. As we know, the increased acetylation of the NF- κ B p65 subunit has been linked to the activation of NF- κ B, which was a crucial transcript factor in inflammatory responses, and NF- κ B activation can trigger the expression of pro-inflammatory cytokines, like TNF- α [34]. Recently, SIRT1 has been suggested to directly act on NF- κ B, and to decrease the acetylation of p65 (Lys310), thereby suppressing the transcript activity and pro-inflammatory expressions [35,36]. At the same time, the activation of AMPK and SIRT1 can alleviate the microglial cell-mediated neuroinflammation, as demonstrated by Velagapudi et al. [37]. From our models, a significant decrease was detected in the protein expression of NF- κ B p65 in DHM-treated AD rats. In agreement, the memory deficits and the increased inflammatory mediators, such as TNF- α and IL-1 β , were observed by Gao et al. in D-gal-induced AD rats, which was closely associated with SIRT1/NF- κ B signaling pathway [38], further indicating

that DHM may ameliorate the cognitive functions and inhibit the neuroinflammatory responses through activation of AMPK/SIRT1 signaling pathway to down-regulate NF- κ B p65.

At the same time, the decreased Bcl-2 (anti-apoptotic protein) and increased Bax (pro-apoptotic protein) were identified in AD rats induced by A β_{1-42} but were reversed after treatment with either 100 or 200 mg/kg of DHM. TUNEL staining further confirmed a significant elevation in the hippocampal apoptosis of AD rats, which was rescued after DHM administration, indicating that DHM may alleviate neuronal injuries and hippocampal apoptosis of AD rats. As reported, neuronal apoptosis is a key cause for AD, and down-regulation of Bcl-2 has been thought to be related to tangle-bearing neurons in AD brains [39,40]. Kou et al. [41] also noted that DHM can decrease the p53-mediated cell apoptosis through miR-34a-mediated SIRT1/mTOR signaling pathway, resulting in the down-regulation of Caspase-3 and promotion of Bcl-2. It has been well-recognized that p53 is not only a common target of SIRT1 [42], but also a critical transcript factor, and its activity enhancement mediates the cell apoptosis [43]. In light of this, we supposed that DHM may activate the AMPK/SIRT1 signaling pathway to improve the neuronal apoptosis possibly through the inhibition of p53-mediated apoptosis signaling pathway.

In conclusion, DHM can activate the AMPK/SIRT1 pathway to inhibit the inflammatory responses and hippocampal neuronal apoptosis, and ameliorates learning deficits of AD rats, which provides novel targets for the clinical treatment of AD.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

Ping Sun designed the study, Jun-Bo Yin and Chun-Xia Wang conceived and supervised the study, Li-Hua Liu performed the examination and the analysis, Jian Guo performed the statistical analysis, Sheng-Hai Wang interpreted the results, Chun-Hui Qu drafted the paper. All authors read and approved the final paper.

Abbreviations

A β , amyloid- β ; A β_{1-42} , amyloid- β_{1-42} ; AD, Alzheimer's disease; AMP, ampepin; AMPK, AMP-activated protein kinase; APP, amyloid precursor protein; DHM, Dihydromyricetin; IL, interleukin; NF- κ B, nuclear factor- κ B; PFA, paraformaldehyde; TNF, tumor necrosis factor.

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