

Magnolol acts as a neurorestorative agent in an $A\beta_{1-42}$ -induced mouse model of Alzheimer's disease

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Abstract. Magnolol may have the potential to alleviate the progression of Alzheimer's disease (AD). The present study was conducted to investigate the broader mechanism of action of magnolol in AD pathogenesis. C57BL/6 mice were randomly divided into five groups (n=6 mice/group): i) Control; ii) AD model; iii) 5 mg/kg magnolol + AD model; iv) 10 mg/kg magnolol + AD model; and v) 20 mg/kg magnolol + AD model. A total of 7 days after modeling, the treatment groups were administered different doses of magnolol (5, 10 and 20 mg/kg) by gavage every day, and a Morris water maze test was conducted after 2 months of treatment. The impacts of magnolol on amyloid β ($A\beta$) plaque deposition and neuroinflammation were assessed using Congo red and immunofluorescence staining. Immunofluorescence staining results were supplemented with western blotting and reverse transcription-quantitative PCR to ascertain the role of magnolol in other pivotal pathological mechanisms, including the formation of intracellular neurofibrillary tangles, compromised synaptic plasticity, and astrocyte and microglia activation. Administration of magnolol effectively mitigated cognitive impairment, reduced $A\beta$ plaque deposition and inhibited neuroinflammation in $A\beta_{1-42}$ -induced mice. Moreover, hippocampal levels of tau, phosphorylated (p-) tau, glycogen synthase kinase 3 β (GSK3 β), p-GSK3 β , synaptophysin, brain-derived neurotrophic factor, glial fibrillary

acidic protein and ionized calcium binding adaptor molecule 1 revealed that magnolol also limited neurofibrillary tangle formation, repaired synaptic plasticity, and inhibited astrocyte and microglia activation. In conclusion, the present findings broaden the current understanding of the mechanisms explaining the neuroprotective effects of magnolol against AD progression. Notably, it may inhibit multiple manifestations of AD, including plaques and neuroinflammation, while also exhibiting the capacity to restore AD-related neurological damage.

Introduction

Continuous improvements in living standards and increasing average lifespan have led to a corresponding rise in the incidence of neurodegenerative diseases, for which aging is the primary risk factor (1). Alzheimer's disease (AD), the most common type of dementia, is a progressive neurodegenerative disease among the elderly population (2). It is estimated that by mid-century, the number of Americans aged ≥ 65 years with AD may grow to 13.8 million (3). This represents a steep increase from the estimated 5.8 million Americans aged ≥ 65 years who have AD today (3). Patients diagnosed with AD experience gradual deterioration of memory and cognitive function, accompanied by a range of behavioral disturbances and neuropsychiatric symptoms (4,5).

Pathological characteristics of AD involve numerous aspects, such as tau phosphorylation, and abnormal expression of glycogen synthase kinase 3 β (GSK3 β), synaptophysin, brain-derived neurotrophic factor (BDNF) and inflammatory cytokines (6-10). Another notable characteristic is excessive amyloid β ($A\beta$) accumulation outside neurons, eventually resulting in the formation of plaques (11). This phenomenon has long been understood to indicate that abnormal amyloidogenic processing is a significant element of AD development (12). However, research has suggested that numerous elderly individuals have $A\beta$ deposits in their brains without exhibiting AD symptoms, suggesting that the deposition of $A\beta$ plaques is not a sufficient condition for the occurrence of AD (13). By contrast, the distribution and severity of neurofibrillary tangles appear to have a stronger association with cognitive dysfunction than $A\beta$ plaque deposition (14). At present, no successful

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Abbreviations: AD, Alzheimer's disease; $A\beta$, amyloid β ; GSK3 β , glycogen synthase kinase 3 β ; BDNF, brain-derived neurotrophic factor; MWM, Morris water maze; PFA, paraformaldehyde

Key words: AD, $A\beta$ plaque, magnolol, neuroinflammation, neurofibrillary tangles, synaptic plasticity

therapy is available that can cure or significantly slow AD progression, despite extensive research on the etiology of the disorder and considerable investment from the pharmaceutical industry (15). As a result, the underlying mechanisms of AD must be elucidated more comprehensively to develop viable treatment strategies.

Numerous bioactive compounds derived from natural sources have exhibited potential anti-AD effects in both laboratory and clinical studies, such as tanshinone IIA (16), naringin (17) and ellagic acid (18). *Magnolia officinalis* (commonly termed Houpu Magnolia or Magnolia-bark), a species of the genus *Magnolia* belonging to the family Magnoliaceae, is an endangered deciduous tree in China (19). Magnolol, a bioactive component isolated from *Magnolia officinalis*, has been extensively documented to possess diverse biological properties, including anti-oxidative, anticancer and anti-inflammatory effects. For example, magnolol has been shown to possess potential in mitigating oxidative stress in white adipocytes, and is thus being considered a promising strategy for combating obesity (20). It has the ability to inhibit the proliferative and migratory capacities of diverse types of human cancer, including pancreatic cancer (21), cervical cancer (22) and hepatocellular carcinoma (23). Furthermore, the administration of magnolol has been shown to confer advantages in attenuating inflammation in patients with diabetic periodontitis (24). Recently, the neuroprotective potential of magnolol in AD-related pathology has also been uncovered. Wang and Jia (25) reported that magnolol can improve cognitive decline through regulating autophagy and the AMPK/mTOR/ULK1 pathway. Chen *et al* (26) established a mouse model of AD with brain insulin resistance, and demonstrated that magnolol could interact with microRNA-200c to alleviate neuroinflammation. Zhu *et al* (27) found that the magnolol-mediated cAMP/PKA/CREB pathway could significantly mitigate A β -induced neuronal injury in SH-SY5Y neuroblastoma cells. It is widely recognized that the initiation and progression of AD involve a multifaceted biological process encompassing various regulatory mechanisms (28,29). The potential existence of additional regulatory mechanisms underlying the impact of magnolol on the progression of AD is a topic of great interest to researchers.

In the present study, a preliminary investigation was conducted into the effects of varying doses of magnolol on several factors related to AD pathology, including tau phosphorylation, GSK3 β , synaptophysin, BDNF, inflammatory cytokines, as well as the activation of astrocytes and microglia. The present study aims to further enhance the understanding of AD pathogenesis and establish a robust foundation for targeted AD therapy.

Materials and methods

Reagents and antibodies. Magnolol (analytical standard, purity $\geq 98\%$) was provided by Shanghai Yuanye Biotechnology Co., Ltd. Congo red dye and A β_{1-42} were acquired from MilliporeSigma, while the hematoxylin dye was supplied by Beyotime Institute of Biotechnology. Goat serum was obtained from Gibco; Thermo Fisher Scientific, Inc. RIPA lysis buffer, bovine serum albumin, ECL Kit and BCA Protein Assay Kit were obtained from Thermo Fisher

Scientific, Inc. The QIAwave RNA Mini Kit, First Strand Kit and QuantiFast SYBR[®] Green PCR Kit were obtained from Qiagen GmbH. The primary antibodies used in the present study were IL-6 (Abcam; cat. no. ab290735), IL-1 β (Abcam; cat. no. ab315084), tau (Abcam; cat. no. ab254256), phosphorylated (p-)tau (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. MN1020), GSK3 β (Abcam; cat. no. ab32391), p-GSK3 β (Abcam; cat. no. ab75814), synaptophysin (Proteintech Group, Inc.; cat. no. 17785-1-AP), BDNF (Proteintech; cat. no. 28205-1-AP), glial fibrillary acidic protein (GFAP; Proteintech Group, Inc.; cat. no. 16825-1-AP), ionized calcium binding adaptor molecule 1 (Iba1; Proteintech Group, Inc.; cat. no. 10904-1-AP) and GAPDH (Proteintech Group, Inc.; cat. no. 60004-1-Ig). The HRP-conjugated anti-rabbit (cat. no. A21020) and HRP-conjugated anti-mouse (cat. no. A21010) secondary antibodies were obtained from Abbkine Scientific Co., Ltd. FITC-labeled goat anti-mouse secondary antibody (cat. no. ab6785) was obtained from Abcam.

Animal grouping and treatment. Healthy male C57BL/6 mice (age, 10-12 weeks; weight, 25-30 g) with normal locomotor and cognitive functions were purchased from Charles River Laboratories, Inc. Before grouping the mice, visible-platform training was carried out to determine the baseline differences in vision and motivation. Mice with abnormal locomotor abilities were excluded. Subsequently, mice were housed in specific pathogen-free cages under standard laboratory conditions including 22-25°C temperature, 40-55% relative humidity and 12-h light/dark cycle, with free access to water and food. The A β_{1-42} stock solution was prepared at a concentration of 1 mg/ml in sterile PBS and then incubated at 37°C for 5 days to facilitate oligomerization.

A total of 30 C57BL/6 mice were randomly divided into five groups (n=6 mice/group): i) Control; ii) AD model; iii) 5 mg/kg magnolol + AD model; iv) 10 mg/kg magnolol + AD model; and v) 20 mg/kg magnolol + AD model. All surgical procedures were performed under aseptic conditions. The AD model was established as follows: The mice were anesthetized by intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg), and then subsequently secured onto a brain stereotaxic device in a prone position. As previously described (30,31), the hippocampal CA1 region serves a critical role in cognitive functions such as learning and memory, and damage or lesions in this region are closely related to cognitive impairment in AD. Additionally, bilateral injection can simulate the pathological changes of diseases more comprehensively, improving the stability and reliability of the model (32). Therefore, the bilateral hippocampal CA1 region was selected as the targeted injection area. The specific coordinate position was 2.4 mm dorsoventral, 0.2 mm anteroposterior and 1 mm mediolateral to the bregma. The aggregated form of A β_{1-42} was administered via intracerebroventricular injection at a volume of 3 μ l and a speed of 1 μ l/min. The injector was left in place for 5 min. Mice in the control group received an equivalent amount of normal saline. To consider animal welfare, animal distress was alleviated as much as possible. A 0.2-ml dose of gentamicin was injected to the suture site for anti-inflammatory treatment when the incision was sutured. Meanwhile, the animals were housed individually and permitted to recuperate from the

anesthesia on a heated mat in order to regulate body temperature at $37.5 \pm 0.5^\circ\text{C}$. After 7 days, the treatment groups were administered varying doses of magnolol (5, 10 and 20 mg/kg) by gavage every day, while mice in the control and AD model groups received an equal volume of normal saline. During the experiments, changes in body weight were monitored once a week, and changes in food and water intake were observed. Although it was not expected, a rapid decrease in normal body weight $>20\%$ was defined as a humane endpoint for the present study. In this study, none of the mice reached the humane endpoint.

A Morris water maze (MWM) test was conducted after 2 months of magnolol treatment. No animals died naturally during the experiments, and all mice were euthanized by overdose with pentobarbital sodium (200 mg/kg; i.p.). The death was confirmed by cardiac and respiratory arrest, along with the absence of response to tail clamping. A total of 3 mice were randomly selected from each group, and the hippocampal tissues were collected for Congo red and immunofluorescence staining. The hippocampal tissues of the remaining mice were stored at -80°C until use. The whole experiment lasted 73 days and a schematic diagram of the experimental protocol is shown in Fig. 1A. The animal experiments were conducted in compliance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (33) and received approval from The Ethics Committee of The School of Medicine of Jinhua Polytechnic (Jinhua, China; approval no. AL-JSYJ202334).

MWM test. As previously described (25), the MWM test was applied to evaluate the spatial memory of the mice. A circular pool with a height of 50 cm and a diameter of 150 cm was used. The water was filled to a depth of 30 cm, while maintaining a temperature of 24°C . The swimming pool was artificially partitioned into four quadrants, designated as quadrant I, II, III and IV. During the 5-day spatial navigation test, a round platform with a diameter of 10 cm was positioned at the center of quadrant IV and submerged 1 cm below the water surface, followed by removing it when the 1-day probe task was conducted. The mice were each subjected to two tests per day, with a minimum interval of 15 min between tests. Each mouse was introduced into the pool and given 60 sec to seek the platform. The time taken to locate the concealed platform was recorded as escape latency (sec). On day 6, the hidden platform was removed to evaluate the memory ability. Subsequently, the mice were permitted to swim freely for 60 sec to seek the removed platform. A video tracking system (version 3.0; SMART, Panlab) was employed to record the distance to target, escape latency and swimming speed.

Section preparation. The hippocampal tissues were collected and promptly fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Subsequently, the specimens were immersed in a solution of 30% sucrose-PFA until they sank to the bottom. The samples were then embedded in paraffin and flash-frozen using liquid nitrogen. Slices with a thickness of $4\ \mu\text{m}$ were obtained using a cryostat (Leica Microsystems GmbH), stored at -80°C , and used for Congo red and immunofluorescence staining.

Congo red staining. Congo red staining was used for A β plaque visualization in the CA1 region of the hippocampus, as previously reported (34,35). The sections underwent deparaffinization and hydration with distilled water, followed by staining in a solution of 0.5% Congo red for 20 min at room temperature. Subsequently, the sections were rinsed in distilled water and rapidly differentiated in an alkaline alcohol solution. The sections were then washed in tap water for 1 min, followed by counterstaining with hematoxylin for 30 sec at room temperature and subsequent rinsing again in tap water for 2 min. Finally, the sections were dehydrated and mounted using resinous mounting medium. For semi-quantification of Congo red staining, images were captured from three hippocampal sections per sample, and Congo red-positive cells were counted manually from the CA1 region of the hippocampus using five random regions, under an optical microscope (scale bar, $50\ \mu\text{m}$; Olympus Corporation). The number of A β plaques was represented by the number of Congo red-positive cells.

Immunofluorescence staining. The tissue slices underwent deparaffinization in xylene for 10 min at room temperature and rehydration with descending concentrations of ethanol (100, 95 and 70% for 3–5 min each), followed by antigen retrieval in heated sodium citrate at 80°C for 25 min. Subsequently, the slices were blocked with 4% goat serum for 15 min at room temperature, after which, the slices were incubated overnight at 4°C with the following primary antibodies: IL-1 β , IL-6, p-tau, Iba1 and GFAP (all diluted to 1:50). After three washes with PBS, the slices were incubated with FITC-labeled goat anti-mouse secondary antibody (1:200) for 1 h in the dark at room temperature. DAPI was used for nuclear staining at room temperature for 15 min. For the expression determination, images were captured from three hippocampal sections per sample. The IL-1 β -, IL-6-, p-tau-, Iba1- and GFAP-positive cells were evaluated under a fluorescence microscope (Olympus Corporation; scale bar, $100\ \mu\text{m}$) from five different CA1 regions of hippocampus. Image-Pro-Plus (version 6.0; Media Cybernetics) was adopted to analyze the fluorescence intensity.

Western blotting. Proteins were extracted from hippocampal tissues using RIPA lysis buffer with protease inhibitors, and their concentration was determined using the BCA Protein Assay Kit. A total of $\sim 25\ \mu\text{g}$ proteins were separated by SDS-PAGE on a 10% gel and subsequently transferred to a PVDF membrane. The membrane was then blocked with 5% bovine serum albumin for 2 h at room temperature before being incubated overnight at 4°C with primary antibodies against tau (1:1,000), p-tau (1:1,000), GSK3 β (1:5,000), p-GSK3 β (1:5,000), synaptophysin (1:20,000), BDNF (1:1,000), GFAP (1:5,000), Iba1 (1:1,000) and GAPDH (1:50,000). After washing the membranes three times with Tris-buffered saline-Tween-20 (0.05%), the HRP-conjugated secondary antibodies (1:10,000) were added and incubated at room temperature for 1 h. The internal reference used was GAPDH. Finally, the immunoreactive protein bands were visualized using an ECL Kit under a Gel-Proanalyzer (version 4.0; MilliporeSigma).

Isolation of RNA and reverse transcription-quantitative PCR (RT-qPCR). The QIAwave RNA Mini Kit was employed for RNA isolation. Subsequently, cDNA was synthesized using the

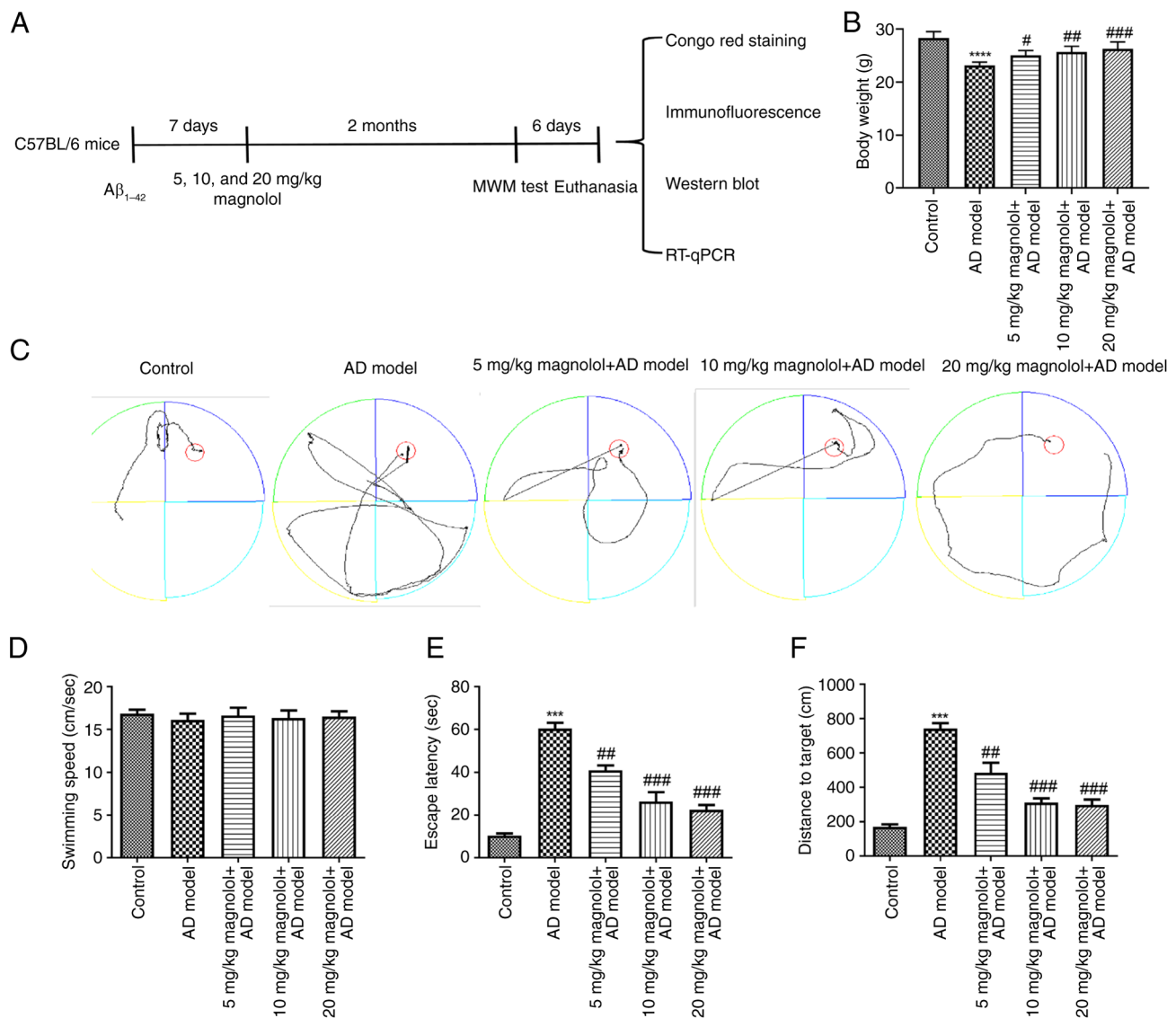


Figure 1. Treatment with magnolol mitigates cognitive impairment in $A\beta_{1-42}$ -induced mice. (A) Schematic diagram of the experimental protocol. (B) Body weight of mice in different groups. (C) Representative tracing graphs of the spatial probe trial. (D) Swimming speed of each group in the MWM test. (E) Escape latency of each group in the MWM test. (F) Distance traveled to the target during the spatial probe test in the MWM test. *** $P < 0.001$, **** $P < 0.0001$ vs. control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. AD model. $A\beta$, amyloid β ; AD, Alzheimer's disease; MWM, Morris water maze; RT-qPCR, reverse transcription-quantitative PCR.

First Strand Kit according to the manufacturer's protocol, and subjected to qPCR analysis using a QuantiFast SYBR Green PCR Kit on the Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 94°C for 10 sec, 60°C for 20 sec and 72°C for 34 sec. Gene expression levels were determined using the $2^{-\Delta\Delta C_q}$ method (36) with GAPDH used as the internal control. The gene primers utilized in this study are detailed in Table I.

Statistical analysis. The data analysis was conducted using SPSS software version 20.0 (IBM Corp.) and the results are presented as the mean \pm standard deviation. Statistical differences among the groups were determined using one-way ANOVA, followed by Tukey's multiple comparisons post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with magnolol mitigates cognitive impairment in $A\beta_{1-42}$ -induced mice. The body weight of mice in different groups was monitored. As shown in Fig. 1B, compared with that in the control group, the body weight of mice with AD was significantly decreased; however, compared with in the AD model group, administration of different doses of magnolol, particularly 20 mg/kg, significantly restored the body weight. The track plots of mice in the MWM test are shown in Fig. 1C. First, the speed of mice swimming in the water maze was assessed to exclude false-positive results. The results revealed that there was no significant difference in the swimming speed of each group of mice (Fig. 1D). Next, it was revealed that the AD model group spent more time to reach the platform compared with the control group in the spatial navigation test (Fig. 1E). However, administration of different doses of

Table I. Reverse transcription-quantitative PCR primer sequences.

Gene	Sequence (5'-3')
Synaptophysin	Forward: GACGTTGGTAGTGCCTGTGA Reverse: GCACAGGAAAGTAGGGGGTC
BDNF	Forward: CGGAGAGCAGAGTCCATTGAG Reverse: CCAGTATACCAACCCGGAGC
GFAP	Forward: CCTGCCAGCTCTCCCT Reverse: AAAGGTGTGGCTGAAATGCG
Iba1	Forward: TGAGGAGATTCAACAGAAGCTGA Reverse: AGACGCTGGTTGTCTTAGGC
GAPDH	Forward: AAGAGGGATGCTGCCCTTAC Reverse: TACGGCCAAATCCGTTTACA

BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule 1.

magnolol (5, 10 and 20 mg/kg) significantly ameliorated the effects of A β ₁₋₄₂ on escape latency. In the spatial probe test, the memory retention of the platform location performed on the day following the spatial navigation test was assessed. It was shown that compared with the mice in the AD model group, magnolol-treated AD mice showed improved spatial learning and memory ability. This was reflected by significant progressive reductions in distance traveled to the target platform (Fig. 1F).

Magnolol reduces A β plaque deposits and inhibits inflammation in A β ₁₋₄₂-induced mice. The accumulation of A β plaques is a crucial characteristic feature of AD. Therefore, the hippocampal tissues were subjected to Congo red staining for A β plaque deposition detection. Sections with A β plaque accumulation displayed a red-colored Congo stain in the CA1 region of the hippocampus. As shown in Fig. 2A, almost no Congo red-positive cells were observed in the control group, indicating that there was almost no accumulation of plaques in the hippocampal CA1 region of the control mice. Hippocampal sections from A β ₁₋₄₂-induced mice in the model group showed more dense Congo red stains, indicating that A β plaques had accumulated. Administration of magnolol, specifically at a dosage of 20 mg/kg, notably reduced the number of Congo red-positive cells in hippocampal tissues and indicated that treatment with magnolol significantly inhibited the accumulation of A β plaques (Fig. 2B). After activation with plaque accumulation, astrocytes and microglia release a diverse array of pro-inflammatory cytokines that lead to impaired neuronal function in the hippocampus, which is crucial for learning and memory formation (37). Immunofluorescence staining was conducted to evaluate the expression of IL-1 β and IL-6 in the hippocampus. It was indicated that relative expression levels of IL-1 β and IL-6 were notably enhanced in the model group, which was in contrast to the control group (Fig. 2C and D). However, treatment with varying doses of magnolol significantly decreased IL-1 β and IL-6 levels in the hippocampus of A β ₁₋₄₂-induced mice.

Magnolol decreases the expression of tau, p-tau, GFAP and Iba1, and restores the levels of GSK3 β , p-GSK3 β , synaptophysin and BDNF. The occurrence of AD involves numerous

crucial pathological mechanisms, such as the formation of intracellular neurofibrillary tangles, disrupted synaptic plasticity, and the activation of astrocytes and microglia (38-40). Therefore, an investigation was conducted into the expression of pertinent factors. As illustrated in Fig. 3A, in comparison with the control group, the protein levels of tau, p-tau, GFAP and Iba1 were increased in the model group, whereas GSK3 β , p-GSK3 β , synaptophysin and BDNF levels were decreased. However, treatment with magnolol, specifically at a dosage of 10 or 20 mg/kg, significantly decreased the protein levels of tau, p-tau, GFAP and Iba1, and restored the levels of GSK3 β , p-GSK3 β , synaptophysin and BDNF in A β ₁₋₄₂-induced mice. RT-qPCR analysis detected the mRNA expression levels of synaptophysin, BDNF, GFAP and Iba1 (Fig. 3B), while immunofluorescence staining assessed p-tau, Iba1 and GFAP expression (Fig. 3C and D), and the results provided further validation for the aforementioned findings.

Discussion

A β ₁₋₄₂ has been widely employed to induce animal models of AD due to its neurotoxic effects, including disruption of calcium homeostasis, neuroinflammation, induction of cell death and mitochondrial dysfunction (41). As previously described (42,43), the dosage of A β administered in the present study elicits a comprehensive spectrum of AD symptoms within 7 days. The hallmark manifestations of AD encompass cognitive decline and memory loss. Previous research has frequently utilized the MWM test to evaluate long-term spatial memory (44,45). In the present study, the findings indicated that magnolol-treated mice with AD exhibited notable improvements in cognitive function, as evidenced by a significant reduction in the distance to the target and escape latency assessed using the MWM test. These findings provide evidence that magnolol is an effective bioactive compound for treating AD. There is mounting evidence to indicate that the excessive accumulation of A β in patients with AD or in the APP/PS1 mouse model is strongly associated with cognitive impairment (46,47). The results of the present study lend credence to the previous study (48) suggesting that A β ₁₋₄₂-induced AD mice showed a significant accumulation of A β plaques.

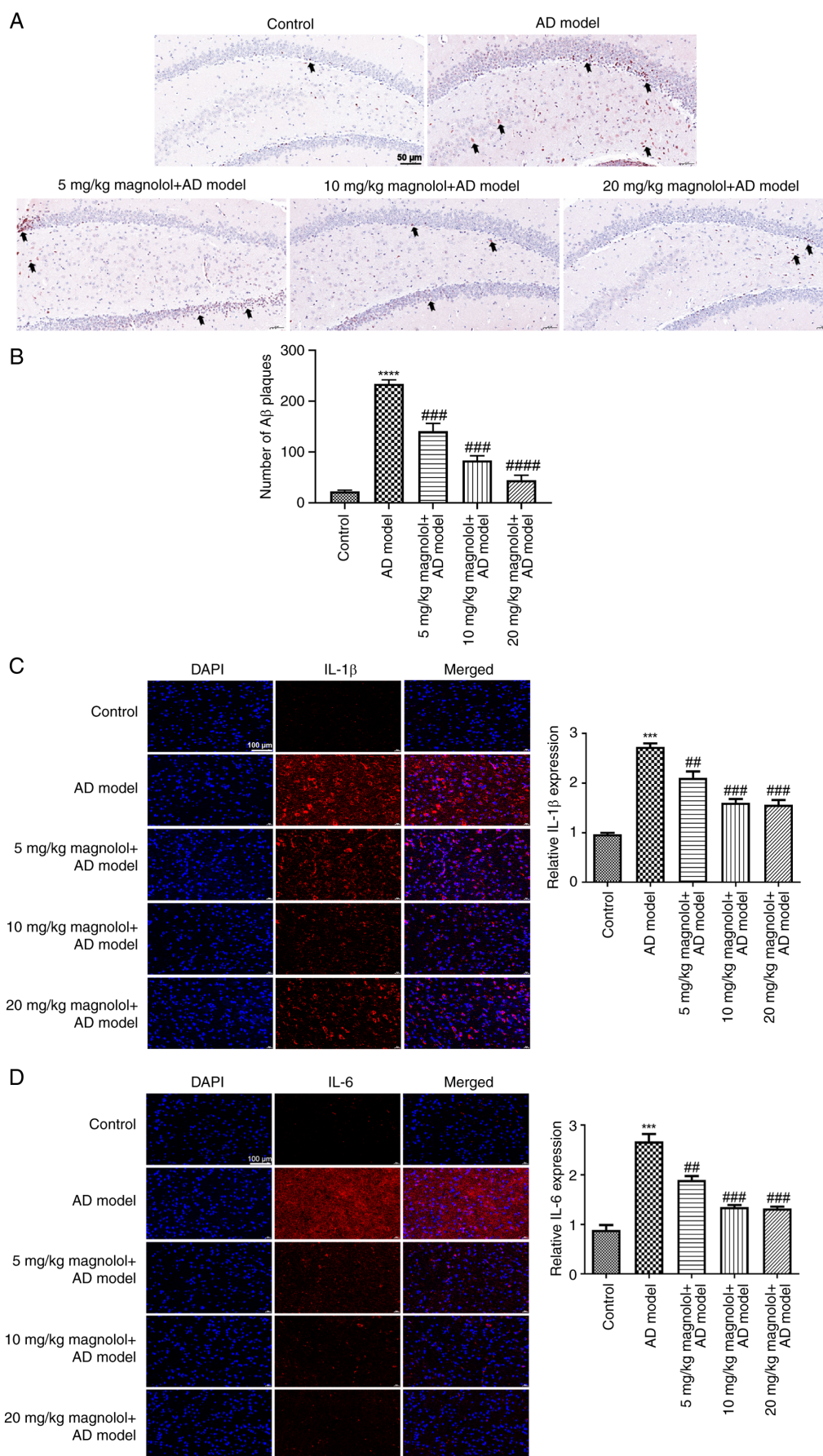


Figure 2. Magnolol reduces A β plaque deposits and inhibits inflammation in A β_{1-42} -induced mice. (A) Images of Congo red-stained A β plaques in the hippocampus. The arrows indicate A β plaques (scale bar, 50 μ m). (B) Quantitative analysis of the number of A β plaques in the hippocampus. The expression levels of (C) IL-1 β and (D) IL-6 in the hippocampus were assessed by immunofluorescence staining (scale bar, 100 μ m). *** P <0.001 and **** P <0.0001 vs. control; ## P <0.01, ### P <0.001 and **** P <0.0001 vs. AD model. AD, Alzheimer's disease; A β , amyloid β .

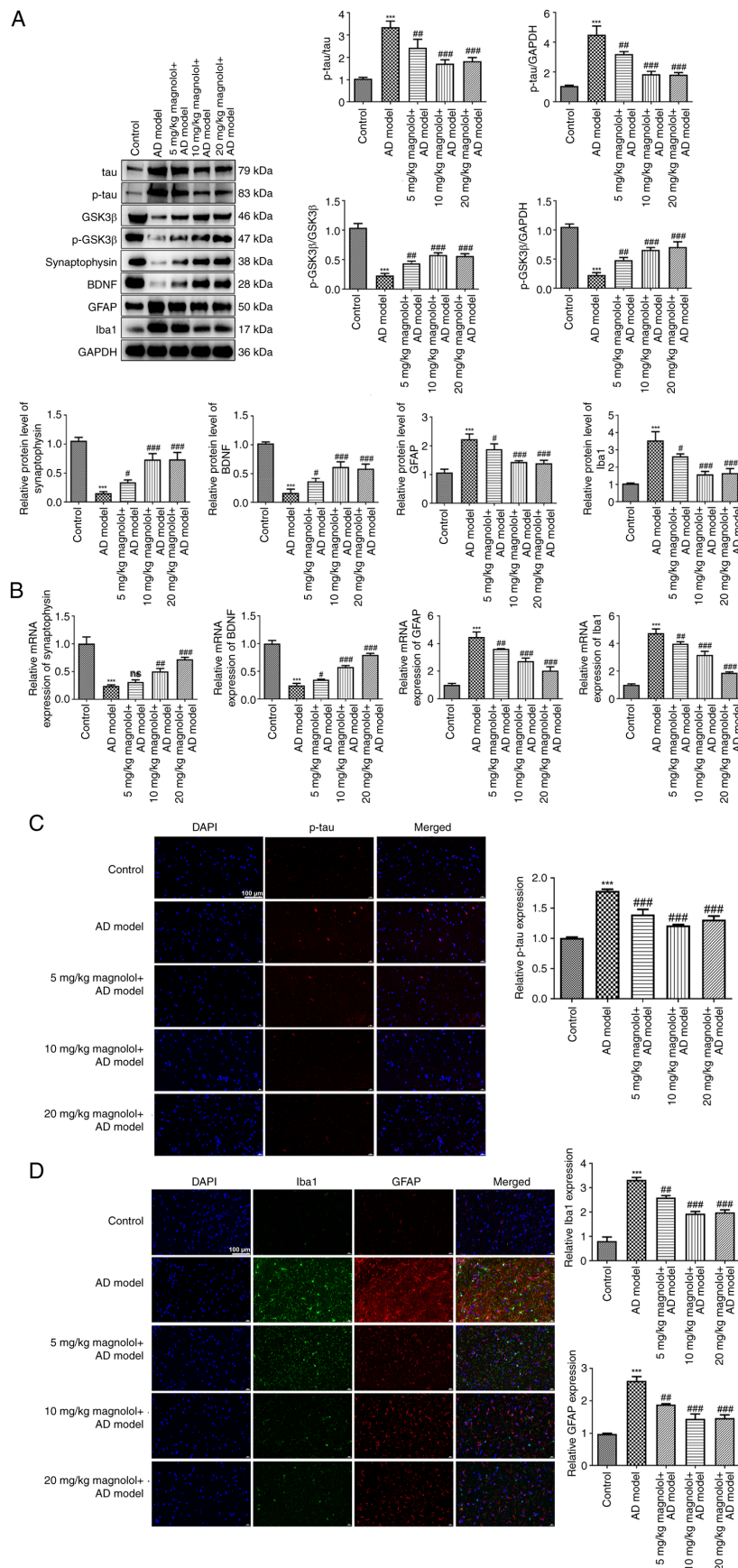


Figure 3. Magnolol decreases the expression of tau, p-tau GFAP and Iba1, and restores the levels of GSK3β, p-GSK3β, synaptophysin and BDNF. (A) Protein levels of tau, p-tau, GSK3β, p-GSK3β, synaptophysin, BDNF, GFAP and Iba1 in each group were determined by western blotting. (B) mRNA expression levels of synaptophysin, BDNF, GFAP and Iba1 in each group were detected by reverse transcription-quantitative PCR. Expression levels of (C) p-tau, (D) Iba1 and GFAP in each group were analyzed by immunofluorescence staining (scale bar, 100 μm). ***P<0.001 vs. control; *P<0.05, **P<0.01, ***P<0.001 vs. AD model. ns, not significant; AD, Alzheimer's disease; GSK3β, glycogen synthase kinase 3β; BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule 1; p, phosphorylated.

Notably, 20 mg/kg magnolol significantly reduced the formation of A β plaques. Additionally, compelling evidence suggests that inflammation caused by A β may lead to cell apoptosis and impaired neuronal function, playing a crucial role in the pathogenesis of AD (49). Therefore, as a potent therapeutic agent for AD, magnolol may also exhibit anti-inflammatory properties during AD progression. As hypothesized, the results of the present study confirmed that the administration of magnolol effectively inhibited the expression of inflammatory cytokines (IL-1 β and IL-6) in the hippocampus of A β ₁₋₄₂-induced mice. These findings may provide evidence supporting the previous results that magnolol can effectively alleviate the symptoms of AD by inhibiting A β deposits and neuroinflammation.

The development of AD also involves a multitude of other crucial pathological mechanisms, including the formation of intracellular neurofibrillary tangles, compromised synaptic plasticity, and the activation of astrocytes and microglia (38-40). Tau, accounting for 80% of the proteins associated with microtubules, serves a crucial role in maintaining the stability of axonal transport tracks and microtubules, while hyperphosphorylation of tau represents a primary factor contributing to the formation of neurofibrillary tangles (50). Tau can undergo phosphorylation at several AD-related sites through the activation of GSK3 β , which is one of the direct causes of tau hyperphosphorylation and can eventually cause spatial memory deficit (51). At the neuronal level, it has been documented that the absence of GSK3 β may lead to a decrease in the stability of dendritic spines and disrupted synaptic transmission within a particular region of the hippocampus (CA1 subset) and cortices, potentially resulting in adverse effects during the progression of AD (52). Learning and memory in the central nervous system rely on synaptic plasticity at a molecular level, and abnormal synaptic function is closely linked to cognitive decline in AD (53). The loss of GSK3 β in the dentate gyrus of mice has been shown to inhibit synaptic transmission in the hippocampus through reducing synaptophysin expression (54). As a member of the neurotrophin family, elevated levels of BDNF have been reported to be associated with synaptic transmission and synaptic plasticity (55). Furthermore, neurodegenerative conditions, including AD, are associated with the activation of astrocytes and microglia. However, research has shown that inhibiting their activities can effectively mitigate neurodegeneration (56). In the present study, given the inhibitory role of magnolol against A β deposits and neuroinflammation in A β ₁₋₄₂-induced mice, the effects of magnolol on these pathological processes were further evaluated by assessing the levels of associated proteins or markers. It was demonstrated that moderate and high doses of magnolol exhibited a more potent inhibitory effect on the levels of tau and p-tau, while also leading to a more pronounced restoration of GSK3 β and p-GSK3 β expression. The findings indicated that magnolol may mitigate the progression of AD by inhibiting the accumulation and hyperphosphorylation of tau as well as activating GSK3 β . Concurrently, the administration of magnolol was found to increase synaptophysin and BDNF levels, indicating that magnolol may exert a reparative effect on synaptic plasticity. Moreover, by reducing the levels of activated astrocyte-specific marker (GFAP) and activated microglia-specific marker (Iba1), it was indicated that magnolol could effectively

suppress the activation of astrocytes and microglia, thereby mitigating the development of AD.

The present study has certain limitations. First, experiments on the role of magnolol in *in vitro* experiments, such as in immortalized mouse hippocampal cells, are needed. Second, elaborating on the inter-relationships between proteins/markers related to key pathological mechanisms of AD and how magnolol affects the interactions between these mechanisms in the present study could be more rigorous. To attain this goal, more elaborate experiments are required. For example, to evaluate how magnolol affects the activation of microglia and astrocytes, double-labeling immunofluorescence analyses of A β deposits and microglia marked with IL-1 β and CD11b, as well as double-labeling immunofluorescence analyses of A β deposits and astrocytes marked by GFAP should be performed. In addition, immunofluorescence staining for microtubule associated protein 2 and Golgi staining for the detection of dendritic spine densities in the CA1 region could be used to assess synaptic plasticity. Third, aside from A β ₁₋₄₂-injected AD mouse models, genetic models of AD, such as APP/PS1 transgenic mice, is another important animal model to study the pathological mechanism of AD. Future studies aim to investigate the more precise and detailed mechanisms of magnolol in this transgenic mouse model. Furthermore, there were some limitations with the current experimental method. First, introducing well-known positive drugs for treating memory deficits in AD model mice, such as memantine, and comparing their effects with those of magnolol might be more rigorous. Second, the pathological process of AD also involves apoptosis of hippocampal neurons, and more detailed experimental methods such as Nissl staining are needed to evaluate neuronal damage. Third, evaluating the spatial memory of mice through MWM test alone is not sufficient, and more classic behavioral and memory tests, such as the open field behavior test, Y-maze test and novel object recognition test, should be performed to assess the locomotor ability, short-term spatial memory and recognition memory, respectively. These experimental methods will be used in future studies.

In conclusion, the present study reveals a broad mechanism of action for magnolol as a neuroprotective agent in the progression of AD. Magnolol exhibited potential in inhibiting A β plaques and neuroinflammation, as well as in reducing the formation of neurofibrillary tangles, restoring synaptic plasticity, and inhibiting the activation of astrocytes and microglia. The findings delineated in the present report could offer crucial data and perspectives for potential clinical interventions for individuals suffering from AD.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LW and MD made substantial contributions to the conception and design of the study. QY, YS, YW, RL and HZ made substantial contributions to the acquisition, analysis and interpretation of the data. QY drafted the manuscript. All authors critically revised the manuscript for intellectual content. LW, MD, and QY confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by The Ethical Committee of The School of Medicine of Jinhua Polytechnic (Jinhua, China; approval no. AL-JSYJ202334).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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