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Biatractylolide Reduced Amyloid Beta Protein-Induced Memory Impairment in Rats

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ABSTRACT

Biatractylolide is a Chinese medicinal compound used to treat various diseases. However, the molecular mechanisms of biatractylolide in preventing and protecting Alzheimer's disease (AD) remained elusive. This study was conducted to observe the effect of biatractylolide on the pathological changes of amyloid beta protein (Aβ)-induced AD. In vitro assays (MTT and flow cytometry) were applied to detect the effect of biatractylolide on PC12 cell proliferation, growth inhibition rate and apoptosis. In order to assess the spatial learning and memory abilities of AD rats, Morris water maze model was applied in vivo, and the activity of the NF-κB signaling pathway and concentrations of TNF-α, IL-6, and IL-1β were measured. The results demonstrated that biatractylolide can reduce apoptosis in hippocampal cells, prevent and improve the cognitive decline induced by Aβ, prevent the morphological changes in hippocampal nerve cells and can reduce the activation of NF-κB signal pathway in vivo. Taken together, biatractylolide appeared to be a useful agent for the treatment of Aβ-related pathologies in the central nervous system, and can be considered for therapeutic application in AD-affected patients.

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Authors' Contribution
MD supervised the study. TZ and ZL performed expermintal. BY and F-fG helped in experimental work.

Key words
Alzheimer's disease, Biatractylolide, Neuroinflammation, Traditional Chinese medicine

INTRODUCTION

The Alzheimer's disease (AD) is a progressive neurodegenerative disease with insidious onsets (Duits *et al*., 2014) and is one of the most common forms of dementia in the elderly people (Wortmann *et al*., 2012). The AD is mainly characterized by the clinical retrograde amnesia (Zhou *et al*., 2011), which has a serious impact on patients and their families (Dargahi *et al*., 2011). Additionally, the disease is characterized by plaque formation in neurons which is composed of amyloid beta protein (Aβ). Deposition of Aβ can activate microglial and astroglial cells leading to the release of TNF-α, IL-6, and IL-1β in the brain resulting the learning and memory dysfunctions (He *et al*., 2011).

The roots of the *Atractylodes macrocephala* Koidz are being used in the traditional Chinese medicine due to their potential in building up vital energy. It can be used for treating anorexia, abdominal distension and diarrhea. The volatile oil from *Atractylodes macrocephala* Koidz carries obvious inhibitory effect on esophageal cancer cells (please add a reference here). Additionally, the aqueous extract proposes inhibitory effects on *Trichophyton flocculus* and *Nocardia stellatus in vitro*. Owing to its antioxidant properties, it has gastric protective effects, neuroprotective effects and anti-tumor impacts (references are needed here). However, molecular mechanisms underlining these medicinal properties of Biatractylolide are not fully elucidated.

In this study, both *in vitro* and *in vivo* models were established to investigate the impact of different doses of biatractylolide, an extract from *A. macrocephala* Koidz, in preventing and treating AD. Additionally, it was revealed that the mechanistic actions of biatractylolide are attributed to the NF-κB signaling pathway.

MATERIALS AND METHODS

Cell culture

The pheochromocytoma cells (PC12) were cultured in DMED medium with 5% fetal bovine serum and horse serum, and 100U/mL penicillin and streptomycin. All cultures were maintained at 37°C and 5% CO_2.

In vitro

Cells were prepared and treated with various concentrations of biatractylolide. The cells were treated with six conditions: control (no treatment), $A\beta_{25-35}$ (Sigma, USA), Aβ+DH (Donepezil Hcl) (Eisai, China), Aβ+BL (biatractylolide, low dose) (20 μM), Aβ+BM (biatractylolide, medium dose) (40 μM), and Aβ+BH (biatractylolide, high dose) (80 μM).

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The PC12 cells were supplemented with these different concentrations of compounds and cultured for 4h. Afterwards, 2 µg/mL Aβ was added 24h before measuring the readings.

MTT assay

After the culture, 20 µL MTT (5 µg/L) were added to each of the well and cultured for 4h at 37°C. The supernatant was discarded and 150 µL DMSO was added per well. The absorbance (A_{490}) of the supernatant was measured after centrifugation at 4000×g for 10 minutes.

Flow cytometry

In a constant temperature incubator at 37°C with 5% CO_2, PC12 cells were cultured for 24h. The supernatant was discarded and washed twice with PBS solution. Cells were centrifuged for 10 min at 1000×g. Then, the Annexin V binding buffer 400 µL was added to the cells in the dark at 2-8°C and cells were gently shaken for 15 min. A total of 10 µL propidium iodide was added to cells, and shaken gently for 5 minutes in a dark room at 2-8°C. Flow cytometry was performed within 1h of treatment to avoid any artifacts.

Western blotting

In a constant temperature incubator at 37°C with 5%CO_2, PC12 cells were cultured for 24h as described above. The supernatant was discarded and the cells were washed twice with PBS. RIPA lysis buffer (Sigma, 100 µL) was added to each well, and the adherent cells were scraped and shredded into a single-cell suspension. The Bicinchoninic Acid Protein Assay Kit was applied to determine the content of the protein. In the SDS-PAGE, the concentration of the separation gel was kept to 12% and concentration of concentration gel was 4%. The PVDF membrane was incubated with primary antibodies against BACE and Aβ and β-actin at 4°C for overnight. The SDS-PAGE gel was transferred to PVDF membrane and washed with PBS solution for three times. The PVDF membrane was incubated with HRP-labeled secondary antibody at room temperature for 2h and then washed with PBS solution for three times. With β-actin as an internal standard, Gel-Pro-Analyzer software was used to scan and analysis the Western blot images.

Immunofluorescence staining

The cell slides were washed with PBS three times and fixed with 4% paraformaldehyde for 10 minutes. Afterword, cells were washed with PBS three times, 0.3% Triton-X100 solution was added to permeabilize cells at RT for 15-20 min. After three times washing, 3% hydrogen peroxide solution was added and incubated at RT for 15 min. The cells were blocked with 1% fetal bovine serum at RT incubated for 30 min. Slides were then washed with PBS for three times and stained with primary antibody against NF-κBp65 for 30 min at 37°C. The slides were incubated with secondary antibody at 37°C for 30 min and washed with PBS three times. The 90% glycerol was used to mount cells and to be observed under fluorescence microscope.

Animal model construction

Healthy male Wistar rats, weighing 200 g, were divided into five groups each consisted on eight rats. Saline or $A\beta_{25-35}$ was administered to rats in the control or the $A\beta_{25-35}$ group, respectively for 13 days. Aβ+DH was administered orally with doses of 0.1mg/200g for 13 days. Similarly, biatractylolide was administered orally at doses of 0.1 (low), 0.3 (medium), or 0.9 mg/kg (high) for 13 days.

MWM testing

All animal groups were subjected to Morris water maze (MWM) (Blokland *et al*., 2004) training from the 8th day of treatment. After 5 days of training, the test was conducted. The rats in the control group were given an intraventricular injection of 3 µL of saline and the rats in the $A\beta_{25-35}$ group and the biatractylolide group were supplemented with 3 µL $A\beta_{25-35}$ (10 ng/µL). All rats were subjected to the MWM test after 24 h.

Before the start of the experiment, the water was adjusted to 20 °C and a round, solid platform was placed 2cm underwater at the center of the bucket (100 cm in diameter). During the experiment, the rats were randomly placed in the water with their heads facing the wall of the bucket. The rats swam freely to find the solid platform. The video system was placed above the bucket to record the trajectory of the rats. In the first experiment, the rats have searched for the solid platform for 90 s and stayed there for 15 s. In 90 seconds, if the rat can't find a solid platform, it was placed on the platform for 15 s before it was removed.

Training continued for five days and performed twice daily. The average latency time of the platform was determined twice and the average reading was recorded. The time duration that the rats spent hunting for the platform was recorded on the 6th day post-start of the experiment.

Rats were killed after the experiment and the hippocampus was dissected from the whole brain. Tissue was rinsed with cold saline, swabbed with filter paper, weighed and then homogenized with a glass homogenizer in an ice bath at a ratio of 1:15 (weight:volume). Centrifugation was performed at 4000×g for 10 min and

the supernatant was discarded. One part of the hippocampal tissue was cryopreserved, and the other part was used for immune-histochemical staining.

TUNEL staining

In Situ Cell Death Detection Kit was used to detect the apoptosis according to the manufacturer's instructions. Briefly, the paraffin sections were inactivated in H_2O_2 followed by addition of 50μL TUNEL reaction solution and incubation for 60 min at 37°C. Sections were washed three times with PBS solution, 50 μL Converter-POD working solution was added, and incubated at 37°C for 30 min. Hematoxylin nucleus was used to stain the sections and developed by DAB. The sections were observed under a light microscope.

Elisa

Levels of TNF-α, IL-6, and IL-1β in the hippocampal tissue homogenates were determined according to the manufacturer's instructions using biomoledular-specific ELISA Detection Kits (Sigma, USA).

Statistical analysis

GraphPad Prism 5.0 software was used to process all data and images. $P < 0.05$ was set as the threshold for statistical significance.

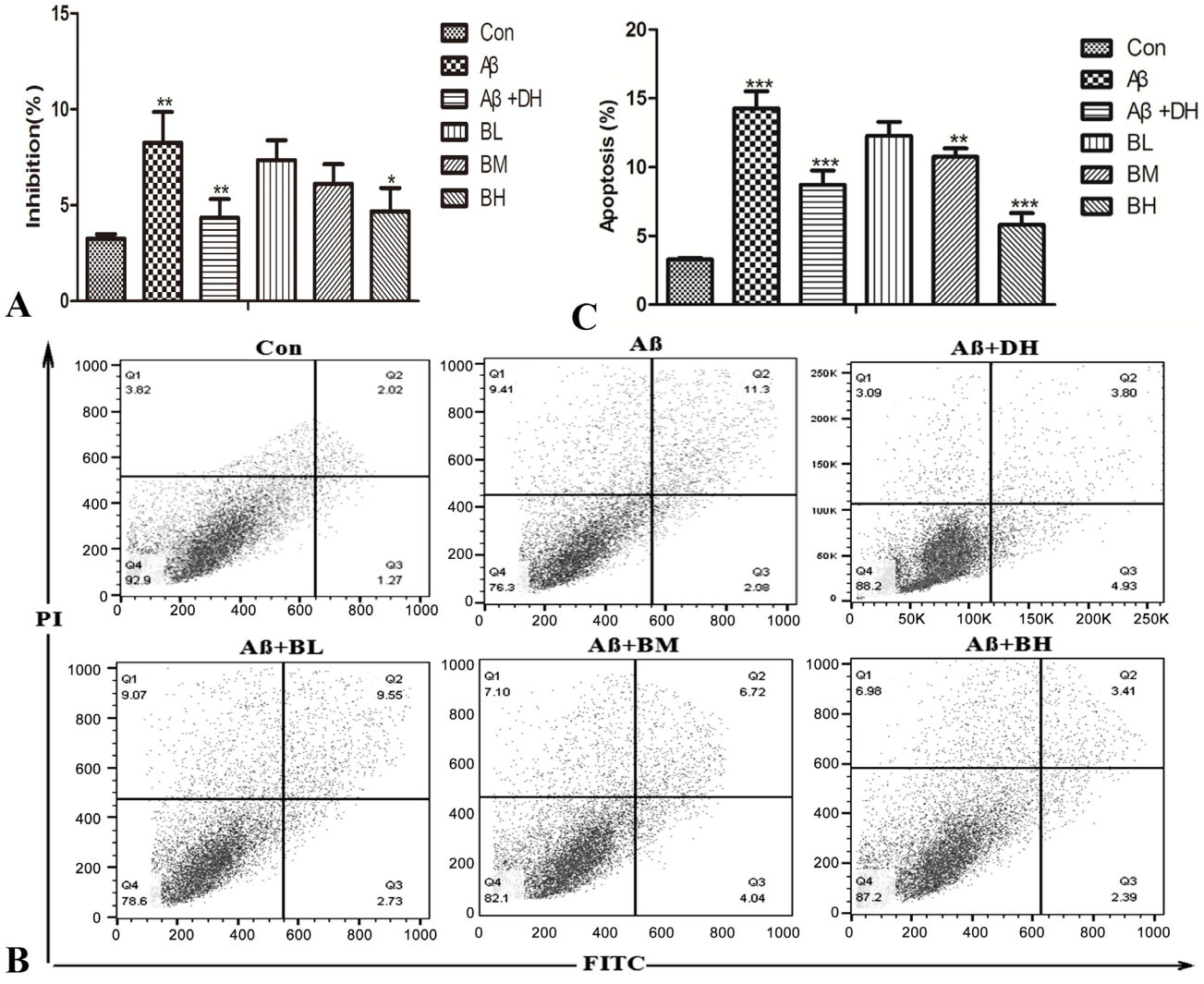

Fig. 1. Biatractylolide alleviated Aβ-induced PC12 cell death.

RESULTS

Biatractylolide attenuates Aβ-induced PC12 cell death

After induction by Aβ, the cell proliferation of the Aβ group increased by 153% compared to the control group (P<0.01; Fig. 1A). Compared to the Aβ group, the inhibition rate of cell proliferation of the Aβ+DH group was reduced by 48% (P<0.05; Fig. 1A), the inhibition rate of cell proliferation of the low dose group was reduced by 11%, cell proliferation inhibition rate of the medium dose group was reduced by 26%, and cell proliferation inhibition rate of the high dose group was reduced by 44% (P<0.05; Fig. 1A). These results demonstrate the fundamental differences between the treatments of Aβ and different concentration of the DHs.

Aβ-induced cell death was further investigated used flow cytometry. The Aβ treatment significantly increased apoptosis (P<0.001; Fig. 1B, 1C), compared with the control group; However, the Aβ+DH group significantly reduced apoptosis compared with Aβ group (P<0.001; Fig. 1B, 1C). The high dose group showed a significantly reduced apoptosis compared to the Aβ group (P<0.001; Fig. 1B, 1C).

After induction by Aβ, the levels of BACE and Aβ were determined by Western blotting (Fig. 2A). As shown, the Aβ and BACE levels were increased by 220% and 675%, respectively in Aβ group (P<0.001 & P<0.001; Fig. 2B) compared to the control group. In comparison with the Aβ group, the levels in the Aβ+DH group were reduced by 69% and 84% (P<0.01 & P<0.001; Fig. 2B). The expression levels were reduced in the low dose group by 16% and 48% (P<0.01 & P<0.001; Fig. 2B). The expression levels in the medium dose group were reduced by 43% and 73% (P<0.01 & P<0.001; Fig. 2B), respectively. Finally, levels in the high dose group were reduced by 67% and 84% (P<0.001 & P<0.001; Fig. 2B), respectively.

Biatractylolide suppresses NF-κB activation in PC12 cells

NF-κBp65 subunit is mainly expressed in the cytoplasm and translocate to nucleus upon activation. The cytoplasmic level of NF-κBp65 in the Aβ group was increased compared to the control group. Interestingly, the donepezil HCl and biatractylolide have reversed this phenomenon. Both donepezil HCl and biatractylolide inhibited the activation of the NF-κB signaling pathway in PC12 cells induced by Aβ (Fig. 2C).

Biatractylolide attenuates Aβ-induced cognitive impairment and hippocampal alterations in rats

In order to investigate the prophylactic effect of biatractylolide on spatial memory impairment induced by Aβ, the MWM test was conducted. Rats in the Aβ group took prominently longer time to reach to the platform than

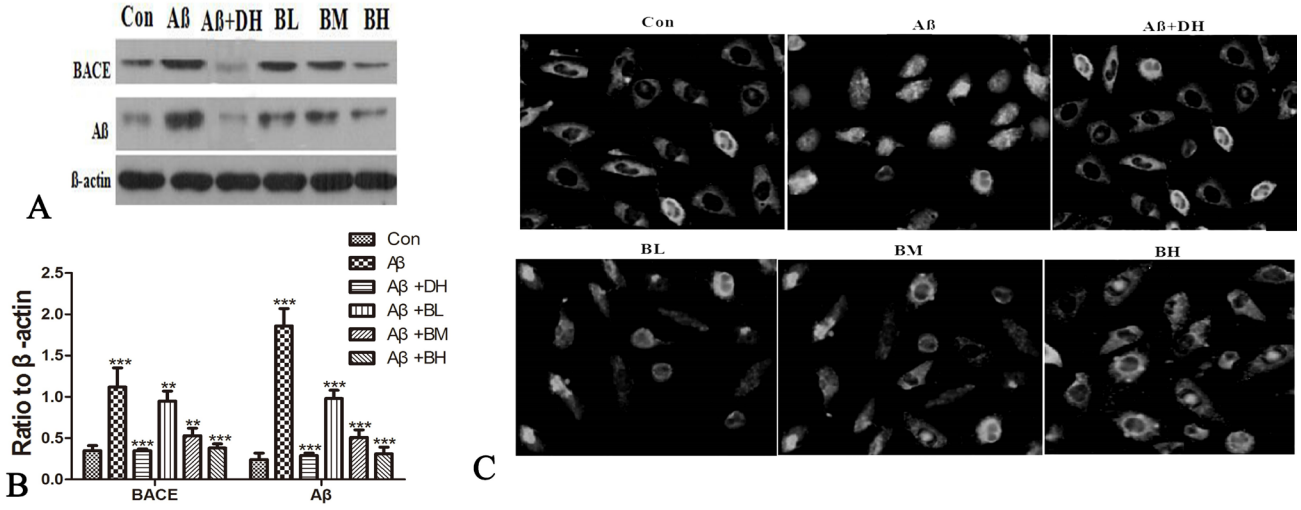

Fig. 2. Effect of Biatractylolide on Aβ-induced changes on levels of Aβ and β-site amyloid precursor protein-cleaving enzyme (BACE).

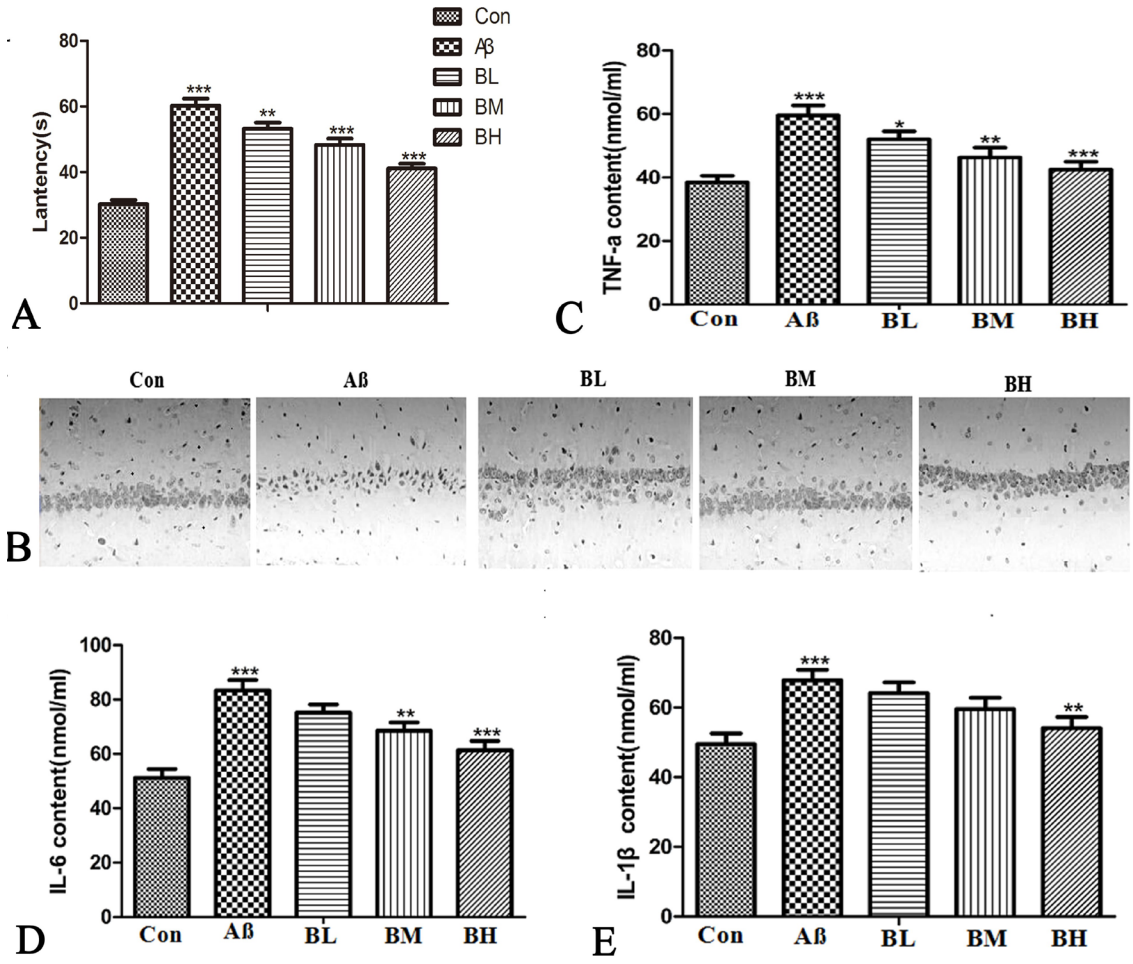

Fig. 3. Biatractylolide attenuate AD-like pathology in rats.

control rats (60.23 ± 1.25 s vs 30.25 ± 1.23 s; P<0.001; Fig.3A). The latency time in the high dose group was prominently less than the Aβ group (41.12 ± 1.48 s vs 60.23 ± 1.25 s; P<0.001; Fig. 3A). The TUNEL assay was used to detect the influence of biatractylolide on apoptosis of the rat hippocampal tissue induced by Aβ. Marked apoptosis and hypocytosis (irregular and loose patterns) were observed in the hippocampal tissue of rats treated with Aβ. These observations were not made in the high dose group as many cells were appeared normal (Fig. 3B). The levels of p-IKBa and NF-κB in the cell nuclei of the Aβ group were increased by 342% and 827% (P<0.001 & P<0.001; Figs. 4C, 4D, 4G), compared to the control group, respectively. On the other hand, the level of IKBa and NF-κB in the cytoplasm decreased by 251% and 260% (P<0.001 & P<0.01; Figs. 4C, 4E, 4F), respectively in Aβ group. The level of Bcl-2 decreased by 54% (P<0.05; Figs. 4A, 4B). The levels of Bax and caspase-3 were increased by 858% and 336% (P<0.001; Figs. 4C, 4D, 4G) in Aβ group, respectively. The levels of p-IKBa and NF-κB in the cell nuclei of the high dose group were reduced by 71% and 73% (P<0.001 & P<0.01; Figs. 4C, 4D, 4G) compared to the Aβ group, respectively. Whereas the levels of IKBa and NF-κB in the cytoplasm were increased by 152% and 191% (P<0.01 & P<0.05; Figs. 4C, 4E, 4F), respectively. The level of Bcl-2 increased by 71% (Figs. 4A, 4B). The level of Bax and caspase-3 decreased by 379% and 405% (P<0.001; Figs. 4A, 4B), respectively. The ELISA was performed to determine the concentrations of TNF-α, IL-6 and IL-1β. Compared to the control group, the concentrations of TNF-α, IL-6, and IL-1β in the rat hippocampus in the Aβ group increased significantly (P<0.001; P<0.001; P<0.001; Figs. 3C, 3D, 3E). Compared to the Aβ group, the concentrations of TNF-α, IL-6 and IL-1β in the high dose group were declined significantly (P<0.001; P<0.001; P<0.01; Figs. 3C, 3D, 3E).

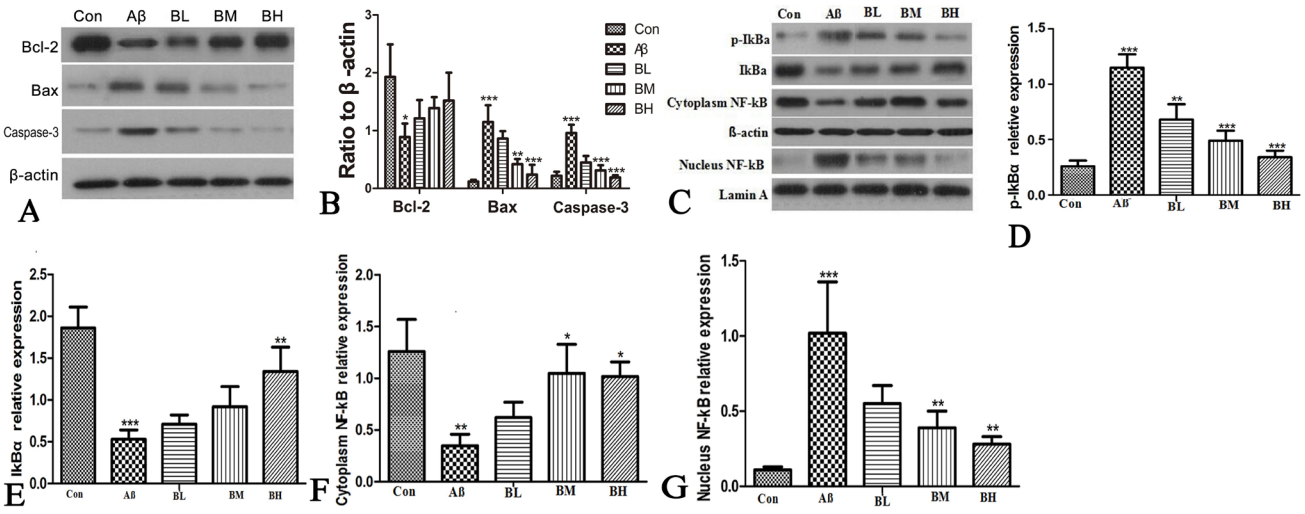

Fig. 4. Biatractylolide attenuate AD-like pathology in rats. The expression of cleaved caspase-3, Bax, and Bcl-2.

DISCUSSION

Biatractylolide is a traditional Chinese medicine and carry various protective and therapeutic effects in diverse diseases. The role of biatractylolide in AD is not clear. Therefore, we explored the AD rat model to investigate some of the mechanisms induced by Aβ. Results showed that biatractylolide effectively improve memory impairment, and limited changes in Aβ, BACE, apoptosis, inflammation, and NF-κB signal transduction levels. To further verify these features, cellular activities and apoptosis were investigated using PC12 cells (Jang *et al*., 2003; Qu *et al*., 2014). We found that biatractylolide prevented both cell proliferation decline and cell death after Aβ- induction. These results demonstrate that biatractylolide protects cells from apoptosis in vitro, and support cells in resisting the effects of Aβ.

The MWM experiment was conducted to assess the learning and memory of experimental animals, which is widely apprehended in senile dementia (Moosavi *et al*., 2012). In this study, we found that biatractylolide effectively prevented learning and memory disorders in rats treated with A*β* (Shi *et al*., 2010).

BACE is a *β*-secretase enzyme and an A*β* precursor (Dong *et al*., 2011) and BACE inhibitors play a key role in the treatment of AD (Zhang *et al*., 2011). In this study, we found that biatractylolide can significantly reduce the level of BACE and A*β*. These results suggest that biatractylolide may be effective in the treatment of AD by inhibiting its progression.

In the mitochondrial pathway, when a drug acts on cells, it enhances the expression level of Bax and inhibits the expression level of Bcl-2, thereby increasing the mitochondrial permeability and promoting cytochrome C release from the mitochondria into the cytoplasm (A Reference is needed here). In turn, this activates the caspase family, and ultimately caspase-3 is activated and cleaved resulting in the DNA rupture and apoptosis. In this study, we found that biatractylolide can reduce the expression level of both Bax and caspase-3 and enhances the expression level of Bcl-2 (Bu *et al*., 2001; Huang *et al*., 2012). These results advocate that biatractylolide has potent anti-apoptotic properties.

Tissue injury or enhanced inflammatory response activates a complex set of cellular biological cascades and results in rapid up-regulation of some inflammatory cytokines (A Reference is needed here). In this study, biatractylolide has reduced TNF-α, IL-6, and IL-1*β* levels in the hippocampus and prevented hyperactive inflammatory responses (Dong *et al*., 2011; Zhang *et al*., 2011; Moreira *et al*., 2016; Wu *et al*., 2012; Saw *et al*., 2013).

When cells are stimulated, IkB is activated, which leads to the phosphorylation of the IkB protein. Upon degradation, NF-κB dimer is released (Gilmore *et al*., 2012) and transferred to the nucleus. In this study, we found that biatractylolide can inhibit the NF-κB signaling pathway (Yang *et al*., 2007) further highlighting the roles of the NF-κB signaling in the biatractylolide-mediated medicinal use.

CONCLUSION

Taken together, biatractylolide inhibits apoptosis in

PC12 cells induced by Aβ, improves neuronal morphology and prevents hippocampal inflammation and cognitive impairment in rats by inhibiting the NF-κB signaling pathway. We also show that biatractylolide has a potential therapeutic role in the treatment and prevention of AD.

Statement of conflict of interest

We confirm that there are no known conflicts of interest associated with this publication.

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