Protective effect of paeoniflorin on inflammation and apoptosis in the cerebral cortex of a transgenic mouse model of Alzheimer's disease

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Abstract. Paeoniflorin, the main active component of the peony plant, exerts various pharmacological effects. Recently, research on the effect of paeoniflorin on the nervous system has gained more attention. The aim of the present study was to determine whether paeoniflorin exerts a protective effect that improves Alzheimer's disease (AD) via inflammation and apoptosis in the cerebral cortex of a transgenic mouse model of AD. Transgenic mice were used to construct the model of AD and were treated with paeoniflorin. The Morris water maze test was used to analyze cognitive function in AD mice. The protein expression levels of nuclear factor-κB, tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and caspase-3 were examined with commercial kits. Expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), phosphorylated (p)-Akt and p-p38 mitogen-activated protein kinase (p-p38 MAPK) in AD were evaluated by western blotting. The neuroprotective effects of paeoniflorin significantly improved cognitive function and ameliorated patterns of escape distance and escape latency in AD mice. Furthermore, the effects of paeoniflorin decreased inflammation and caspase-3 activity, and inhibited cell death via increasing the Bcl-2/Bax ratio and p-Akt expression levels, and downregulating p-p38 MAPK expression in AD mice.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease of the cerebral cortex, which affects the elderly. Its main clinical features include progressive memory impairment, cognitive impairment and reduced quality of life (1). The typical pathological features are extracellular accumulation of β-amyloid (Aβ) in the hippocampus of the brain, the formation of senile plaques, abnormal accumulation of tau protein within brain cells, the appearance of neurofibrillary tangles composed of paired helical filaments, decreased numbers of cerebral cortical neurons, and neocortex and meningeal vascular amyloidosis (2,3).

AD is a chronic degenerative disease of the central nervous system. Previous studies have demonstrated that AD occurrence and development is closely associated with abnormal deposition of Aβ in the brain (4). Abnormal deposition of Aβ results in sustained activation of inflammatory repairing mechanisms. The transformation from acute reaction into chronic inflammatory damage under normal circumstances may be one of the key factors in the pathogenesis of AD (5). Microglia and astrocytes are the main immune cells participating in the central inflammatory cascade of AD (6). The chemokines produced by Aβ-activating astrocytes are potential chemoattractants of microglial cells and macrophages, and also upregulate the expression of inflammatory cytokines, including interleukin (IL)-1 and IL-6 (7). Therefore, the inhibition of Aβ-induced activation of astrocytes may be an important therapeutic strategy for AD, which is caused by the neuropathological changes associated with Aβ (8).

One of the pathological features of AD is the loss of a large number of neurons. The predominant underlying mechanism of neuronal loss resulting from AD is apoptosis, and the neuronal apoptosis hypothesis is an important aspect of AD pathogenesis (9). The neurons of the brain are particularly sensitive to apoptotic damage (10). Factors that induce apoptosis, such as Aβ, oxidative damage and low energy metabolism are present in AD brain tissues (11). A previous study hypothesized that apoptosis is one of the mechanisms underlying the death of AD brain neurons, in which members of the B-cell lymphoma 2 (Bcl-2) family are key in the gene regulation process of apoptosis (12). The Bcl-2 family is divided into two categories: The

anti-apoptotic genes, including Bcl-2; and the pro-apoptotic genes, including Bcl-2-associated X protein (Bax), which is involved in the regulation of apoptosis by activating a series of downstream genes (13).

Paeoniflorin was isolated from the *Ranunculaceae* plant, peony, for the first time in 1963; it is one of the main active components of peony (14). Research into the pharmacological effects of paeoniflorin has identified that paeoniflorin possesses anti-spasm, antipyretic cooling, anti-inflammation, anti-ulcer, anti-oxidation, anti-clotting, and pain and cholesterol regulatory properties (10,15). The underlying mechanisms remain to be elucidated, however, a number of receptors and ion channels have been suggested as possible targets for the pharmacological effects of paeoniflorin (16,17). It has been demonstrated that paeoniflorin may exert an effect on the nervous system and on neurodegenerative diseases, such as AD and Parkinson's disease (18). The present study demonstrated that the neuroprotective effects of paeoniflorin improved AD via inflammation and apoptosis.

Materials and methods

Materials. Paeoniflorin (purity, ≥98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and its chemical structure is presented in Fig. 1. Nuclear factor-κB (NF-κB) p65 unit (cat. no. H202; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), tumor necrosis factor-α (TNF-α; cat. no. E-CL-R0019c; Wuhan Elabscience, Biotechnology Co., Ltd., Wuhan China), IL-1β (cat. no. H002; Nanjing Jiancheng Bioengineering Institute), IL-6 (cat. no. H007; Nanjing Jiancheng Bioengineering Institute) and caspase-3 (cat. no. C1115; Beyotime Institute of Biotechnology, Nanjing, China) commercial kits were purchased. A bicinchoninic acid protein quantification kit was purchased from Sigma-Aldrich (cat. no. BCA1-1KT).

Transgenic mice. The present study was approved by the Institutional Animal Care and Use Committee at Dalian University (Dalian, China). Transgenic mice (n=16; Cyagen Biosciences, Guangzhou, China) expressing the human mutant PS2 and under the control of neuron-specific enolase (NSE) were maintained in the genetic background of C57BL/6 x DBA/2 mice. All mice were maintained in the laboratory for 2 weeks under a 12-h light/dark cycle (housed with the mice of the same group at 23±1˚C with 50% relative humidity), fed a standard laboratory diet and had access to water *ad libitum*.

Animal grouping. Control non-transgenic mice were divided into two groups, as follows: i) The control group (Con; n=8), non-transgenic mice receiving sodium pentobarbital [0.1 ml/100 g administered intraperitoneally (i.p.)]; and ii) the control-paeoniflorin group (Con-Pae; n=8), non-transgenic mice receiving 2.0 mg/kg paeoniflorin for 24 h. Transgenic mice were divided into two groups, as follows: i) AD group (Alz; n=8), transgenic mice receiving sodium pentobarbital (0.1 ml/100 g i.p.); and ii) AD paeoniflorin group (Alz-Pae; n=8), transgenic mice receiving 2.0 mg/kg paeoniflorin for 24 h.

Morris water maze test. Following treatment with paeoniflorin for 24 h, Morris water maze tests were performed, as described in a previous study (19). All the mice were administered the non-visible platform trial twice per day for the first five days, a probe trial on the sixth day, and a visible platform trial on the seventh day. All the mice learned to use visual cues in the room to navigate to an escape platform located at a fixed position and hidden or submerged 1 cm below the surface of the water. All the mice were placed in the pool from different quadrants for training periods of 120 sec. If the mice did not find the platform within 120 sec, the latency was recorded as 120 sec. All mice were replaced on the platform for 20 sec, and the next training period was performed following 120 sec of rest. On each of the five acquisition days, the platform was removed, and the number of crossings of the platform location within 120 sec (crossing number) was recorded.

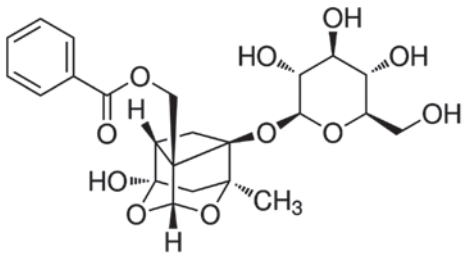

Figure 1. Chemical structure of paeoniflorin.

Evaluation of inflammation and caspase-3 activity. Following treatment with paeoniflorin for 24 h, the mice were sacrificed by cervical dislocation under anaesthesia (pentobarbital), and the cerebral cortex samples were rapidly removed. The cortex samples were snap-frozen on dry ice and stored at -80˚C. The cortex samples were homogenized in physiological saline (0.1 ml/100 g; Dalian Yuanda Pharmaceutical Co., Ltd., Dalian, China) and centrifuged at 12,000 x g for 10 min at 4˚C. The liquid supernatant was collected to analyze the activity of NF-κB p65, TNF-α, IL-1β, IL-6 and caspase-3 activities according to the manufacturer's protocols (Westang Biotech, Co., Ltd.).

Western blot analysis of protein expression levels. Following treatment with paeoniflorin for 24 h, cerebral cortex samples were homogenized with PRO-PREP™ protein extraction solution (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and centrifuged at 12,000 x g at 4˚C for 10 min. The protein concentration was determined using a bicinchoninic acid protein quantification kit. Equal protein quantities (50 μg) were loaded onto a 10% polyacrylamide gel (Beyotime Institute of Biotechnology) for 90 min for electrophoresis (100 V) and subsequently transferred to polyvinylidene fluoride membranes (0.22 mm; EMD Millipore, Billerica, MA, USA). Following blocking of nonspecific binding with Tris-buffered saline (Beyotime Institute of Biotechnology) containing skimmed milk, the membranes were incubated with the following primary antibodies overnight at 4˚C: Bcl-2 (cat. no. sc-578; 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bax (cat. no. sc-20067; 1:1,500, Santa Cruz Biotechnology, Inc.), phosphorylated (p)-Akt (cat. no. sc-293125; 1:1,000; Santa Cruz Biotechnology, Inc.), p38 mitogen-activated protein kinase (p38 MAPK;

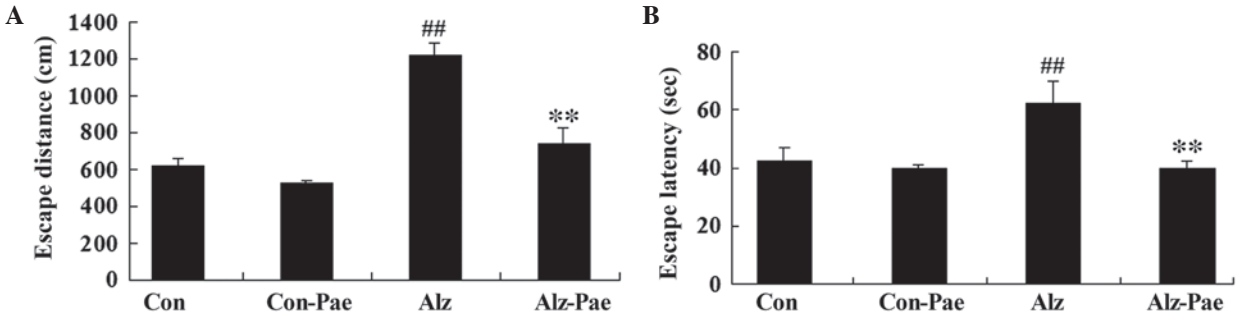

Figure 2. Protective effect of paeoniflorin improves cognitive function in Alzheimer's disease rats. The protective effect of paeoniflorin reduces patterns of escape (A) distance and (B) latency in Alzheimer's disease rats. ##P<0.01 vs. the Con group; **P<0.01 vs. the Alz group. Con, control group; Con-Pae, control paeoniflorin-treated (2.0 mg/kg) group; Alz, Alzheimer's disease group; Alz-Pae, Alzheimer's disease paeoniflorin-treated (2.0 mg/kg) group.

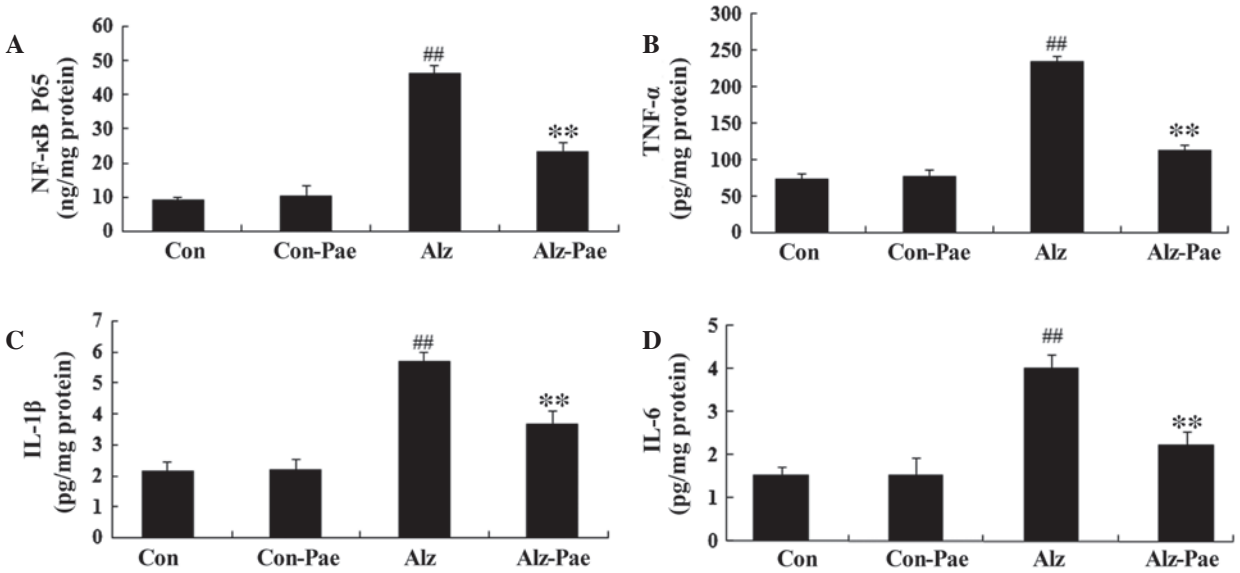

Figure 3. Protective effect of paeoniflorin decreased inflammation in Alzheimer's disease rats. Protective effect of paeoniflorin decreased the (A) NF-κB p65 unit, (B) TNF-α, (C) IL-1β and (D) IL-6 activities in Alzheimer's disease rats. ##P<0.01 vs. the Con group; **P<0.01 vs. the Alz group. Con, control group; Con-Pae, control paeoniflorin-treated (2.0 mg/kg) group; Alz, Alzheimer's disease group; Alz-Pae, Alzheimer's disease paeoniflorin-treated (2.0 mg/kg) group. NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

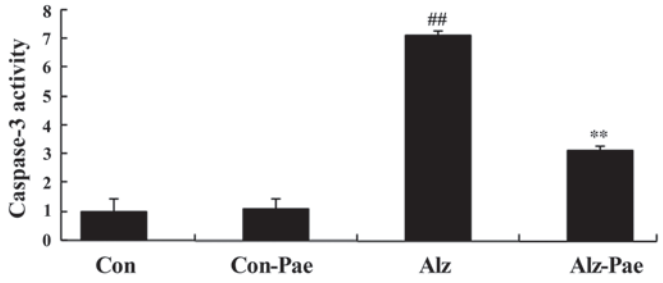

Figure 4. Protective effect of paeoniflorin influences caspase-3 activity in Alzheimer's disease rats. ##P<0.01 vs. the control group; **P<0.01 vs. the Alz group. Con, control group; Con-Pae, control paeoniflorin-treated (2.0 mg/kg) group; Alz, Alzheimer's disease group; Alz-Pae, Alzheimer's disease paeoniflorin-treated (2.0 mg/kg) group.

cat. no. sc-398305; 1:1,000; Santa Cruz Biotechnology, Inc.), p-p38 MAPK (cat. no. sc-7973; 1:1,000; Santa Cruz Biotechnology, Inc.) and β-actin (cat. no. sc-8432; 1:500; Sangon Biotech Co., Ltd., Shanghai, China). The membranes were washed three times with washing buffer (Beyotime Institute of Biotechnology) and incubated for 1 h at 37˚C with secondary antibodies (sc-53804; 1:5,000; Santa Cruz Biotechnology, Inc.). The membrane blots were developed using enhanced chemiluminescence reagents (cat. no. P0018A; Applygen Technologies, Inc., Beijing, China). The band intensity was resolved using a gel image analysis system (Optiquant; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The data are presented as the mean ± standard error and assessed using one-way analysis of variance with a 95% confidence interval. P<0.05 was considered to indicate a statistically significant difference.

Results

Protective effect of paeoniflorin improves cognitive function in AD mice. To investigate whether the protective effect of paeoniflorin improves cognitive function in AD mice, the Morris water maze test was performed. Patterns of escape distance and latency were significantly increased in the

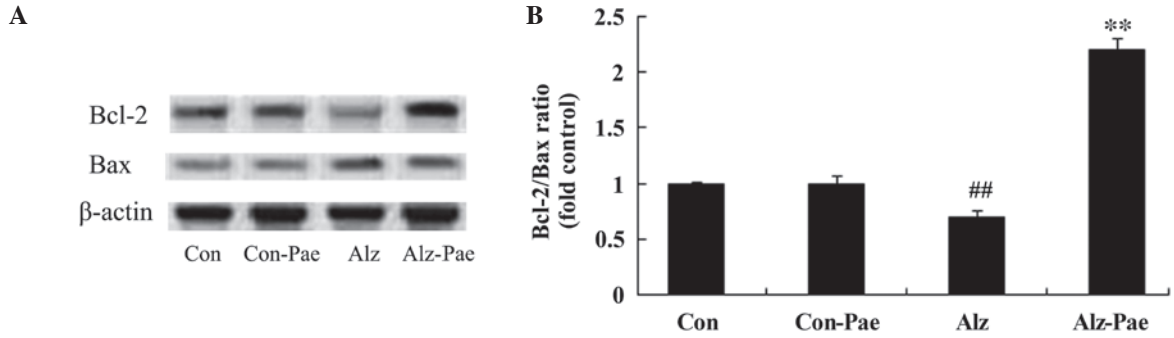

Figure 5. Protective effect of paeoniflorin influences Bcl-2/Bax ratio in Alzheimer's disease mice. Protective effect of paeoniflorin was analyzed using (A) western blotting assays and (B) statistical analysis of Bcl-2/Bax ratio in Alzheimer's disease mice. $^{\#\#}$P<0.01 vs. the Con group; **P<0.01 vs. Alz group. Con, control group; Con-Pae, control paeoniflorin-treated (2.0 mg/kg) group; Alz, Alzheimer's disease group; Alz-Pae, Alzheimer's disease paeoniflorin-treated (2.0 mg/kg) group; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

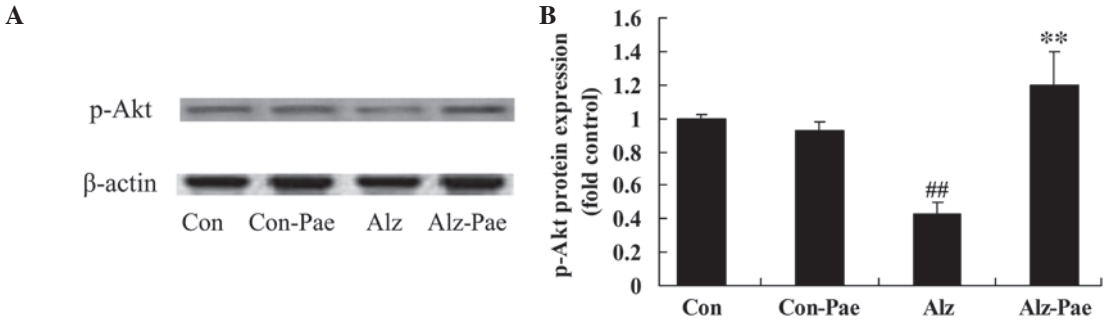

Figure 6. Protective effect of paeoniflorin influences p-Akt in Alzheimer's disease mice. Protective effect of paeoniflorin was analyzed using (A) western blotting assays and (B) statistical analysis of p-Akt protein expression in Alzheimer's disease mice. $^{\#\#}$P<0.01 vs. the Con group; **P<0.01 vs. the Alz group. Con, control group; Con-Pae, control paeoniflorin-treated (2.0 mg/kg) group; Alz, Alzheimer's disease group; Alz-Pae, Alzheimer's disease paeoniflorin-treated (2.0 mg/kg) group.

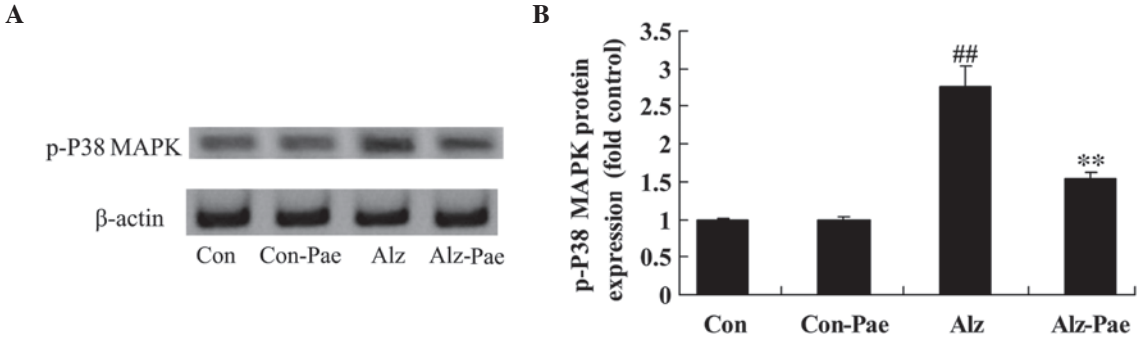

Figure 7. Protective effect of paeoniflorin influences p-p38 MAPK in Alzheimer's disease mice. Protective effect of paeoniflorin was analyzed using (A) western blotting assays and (B) statistical analysis of p-p38MAPK protein expression in Alzheimer's disease mice. $^{\#\#}$P<0.01 vs. the Con group; **P<0.01 vs. the Alz group. Con, control group; Con-Pae, control paeoniflorin-treated (2.0 mg/kg) group; Alz, Alzheimer's disease group; Alz-Pae, Alzheimer's disease paeoniflorin-treated (2.0 mg/kg) group. MAPK, mitogen-activated protein kinase; p, phosphorylated.

transgenic mice, compared with those of the control group (P<0.05; Fig. 2). However, these values were significantly decreased by treatment with paeoniflorin (Alz-Pae), compared with the AD group (Alz; P<0.05; Fig. 2). These results suggest that paeoniflorin may improve cognitive function of transgenic mice.

Protective effect of paeoniflorin decreases inflammation in AD mice. To investigate whether the protective effect of paeoniflorin decreased inflammation in AD mice, the activity of NF-κB p65, TNF-α, IL-1β and IL-6 was analyzed. These inflammatory factors were significantly increased in the Alz, compared with the Con group (P<0.05; Fig. 3). Notably, paeoniflorin treatment (Alz-Pae) significantly decreased the activity of inflammatory factors in the transgenic mice, compared with the Alz group (P<0.05; Fig. 3).

Protective effect of paeoniflorin influences caspase-3 activity in AD mice. To investigate whether the protective effect of paeoniflorin influences caspase-3 in AD mice, caspase-3

activity was analyzed. Caspase-3 activity was significantly increased in the Alz group, compared with the Con group (P<0.05; Fig. 4). Administration of paeoniflorin (Alz-Pae) significantly decreased the caspase-3 activity of transgenic mice, compared with the Alz group (P<0.05; Fig. 4).

Protective effect of paeoniflorin influences the Bcl-2/Bax ratio in AD mice. To investigate the protective effect of paeoniflorin on Bcl-2/Bax ratio in the AD mice, the Bcl-2 and Bax protein expression levels were detected by western blot analysis. Bcl-2 protein expression was significantly suppressed and Bax protein expression was increased in the Alz group, compared with the Con group (P<0.05; Fig. 5A). Paeoniflorin treatment (Alz-Pae) significantly reversed Bcl-2/Bax protein expression in transgenic mice, which exhibited increased Bcl-2 protein expression levels and decreased Bax protein expression levels, compared with the Alz group (P<0.05; Fig. 5A). The Bcl-2/Bax ratio was decreased in the Alz group compared with the Con group, and increased in the Alz-Pae group compared with the Alz group (Fig. 5B).

Protective effect of paeoniflorin influences p-Akt in AD mice. To investigate the protective effect of paeoniflorin on p-Akt in AD mice, p-Akt protein expression levels were evaluated by western blot analysis. The western blots indicate that the expression levels of p-Akt were significantly reduced in the Alz group compared with the Con group (P<0.05; Fig. 6A). However, treatment with paeoniflorin significantly increased p-Akt expression in the Alz-Pae mice compared with the Alz group (P<0.05; Fig. 6B).

Protective effect of paeoniflorin influences p-p38 MAPK in AD mice. To further analyze the protective effect of paeoniflorin on MAPK in AD mice, the p-p38 MAPK protein expression levels were examined by western blot analysis. The results of the western blotting indicated that the p-p38 MAPK protein expression was significantly increased in the Alz group compared with the Con group (P<0.05; Fig. 7). The p-p38 MAPK protein expression was significantly decreased by treatment with paeoniflorin (Alz-Pae), compared with the Alz group (P<0.05; Fig. 7B).

Discussion

AD has a high incidence that will continue to increase due to prolonged average life expectancy, an aging population in China and an increase in the number of elderly individuals (20). Early diagnosis is difficult, there is a lack of effective therapeutic agents and current treatment strategies are not effective, thus AD requires further research. Recent studies have demonstrated that certain types of Chinese medicine may have an effect on AD (21). However, due to the subjectivity, lack of quantitative indicators and clinical difficulty in administration, their therapeutic applications are limited (22). The present study observed that the neuroprotective effect of paeoniflorin significantly improved cognitive function and reduced patterns of escape distance and latency in AD mice. Kapoor (23) reported that the neuroprotective effects of paeoniflorin protect against glutamate-induced neurotoxicity via the Bcl-2/Bax signaling pathway in PC12 cells. Guo *et al* (24) demonstrated that paeoniflorin may be a potential neuroprotective agent for stroke and protected against ischemia-induced brain damage in mice.

In recent years, it has been demonstrated that Aβ protein-induced inflammation may lead to AD pathogenesis (25). AD nerve inflammation is an immune reaction involving the microglia and astrocytes of the brain (26). Activated microglia and astrocytes express a large quantity of inflammatory cytokines, such as TNF-α, IL-1β and IL-6, and specific receptors on the cell surface are involved in inflammation and death of neighboring cells in the brain (27). Aβ proteins accumulate in the brain of patients with AD, which increase the number of receptors on microglial cells. The ligand is more easily integrated into the cell, resulting in nerve cell damage (28). Data from the current study demonstrated that the neuroprotective effects of paeoniflorin significantly decreased the activity of NF-κB p65, TNF-α, IL-1β and IL-6 in AD mice. Sun *et al* (29) indicated that paeoniflorin suppressed inflammation of asthmatic mice, and Jiang *et al* (30) reported that the anti-inflammatory effect of paeoniflorin inhibits systemic inflammation and activation of NF-κB in experimental sepsis.

The occurrence of AD is associated with apoptosis, as abnormal expression of Bcl-2, Bax and caspase-3 are directly involved in apoptosis (31). Bcl-2 inhibits apoptosis to protect cell survival, rather than promoting cell proliferation, by stabilizing the mitochondrial membrane, preventing its release of caspases, apoptosis-associated factors and cytochrome *c* (32,33). The Bcl-2 family includes Bcl-2, which inhibits apoptosis, and Bax, which promotes apoptosis. However, Bax has an inhibitory effect on Bcl-2 and promotes the release of cytochrome *c*, thus activating caspases and accelerating the induction of apoptosis (31). The regulatory effects of Bcl-2 and Bax on apoptosis are in opposition, thus, they are regarded as co-regulators of apoptosis. In the present study, administration of paeoniflorin effectively attenuated the activity of caspase-3 and increased Bcl-2/Bax protein expression levels in the AD mice. Sun *et al* (34) reported that the effect of paeoniflorin protects against glutamate-induced neurotoxicity via Bcl-2/Bax signaling pathways in PC12 cells.

Phosphatidylinositol-3-kinases (PI3K) are important in signal transduction pathways in cells, Akt (also termed protein kinase B) is key in the signaling pathway (35). PI3K/Akt signaling pathways are involved in the regulation of cell apoptosis, proliferation and differentiation, as well as a series of physiological activities and metabolism (36). MAPK is a type of serine and threonine protein kinase in cells, common in a variety of organisms (including yeast and mammalian cells), involved in mediating the growth, development, division, differentiation, death and synchronization of multiple cellular processes (37). In the present study, pretreatment with paeoniflorin increased p-Akt and decreased p-p38 MAPK protein expression levels in AD mice. Xu *et al* (38) demonstrated that paeoniflorin promotes the phosphorylation of Akt and attenuates lipopolysaccharide-induced permeability of endothelial cells. Wankun *et al* (39) indicated that paeoniflorin protects against oxidative stress and suppresses H_2O_2-induced p38 MAPK in human retinal pigment epithelium cells.

In conclusion, the current study demonstrates that the neuroprotective effect of paeoniflorin improves AD via

influencing inflammation and Bcl-2/Bax protein expression in the cerebral cortex of transgenic mice models of AD. In addition, the results suggest that paeoniflorin ameliorated the cognitive dysfunction in AD mice.

Acknowledgements

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