RESEARCH ARTICLE

Baicalin attenuates Alzheimer-like pathological changes and memory deficits induced by amyloid β_{1-42} protein

Chong Chen · Xiaohong Li · Peilong Gao · Yue Tu · Mingliang Zhao · Jianwei Li · Sai Zhang · Haiqian Liang

Abstract Baicalin is one bioactive flavone with anti-inflammatory and neuroprotective activities. The neuroprotective effects of baicalin on pathological changes and behavioral deficits were explored in a mouse model of amyloid β (Aβ) $_{1-42}$ protein-induced Alzheimer's disease (AD). Mice received a bilateral injection of $A\beta_{1-42}$ protein into the hippocampus, then they were treated with baicalin (30, 50 and 100 mg/kg body weight, orally) or Tween 80. The therapeutic effects of baicalin were monitored by Morris water maze trial and probe test. Then mice were sacrificed for immunohistochemistry and western blot analysis. After a relatively short-term treatment of 14 days, 100 mg/kg of baicalin significantly ameliorated memory impairment in the Morris water maze test and probe test, and also attenuated glial cell activations and increase of TNF-α and IL-6 expressions induced by $A\beta_{1-42}$ protein. These results suggest that baicalin ameliorated $A\beta_{1-42}$ protein-related pathology and cognitive dysfunction via its anti-neuroinflammatory activity, and may be a potential candidate for the treatment of AD.

Keywords Alzheimer's disease · Baicalin · Neuroinflammation · Memory · Glial activation

Introduction

Alzheimer's disease (AD) is a multifactorial neurological disorder, clinically characterized by a progressive loss of cognitive function, neuropsychiatric and behavioral disorders (Rong et al. 2013; Wimo et al. 2003). Neuropathological examination of the brains of AD patients reveals massive accumulation of extracellular amyloid plaques composed of aggregated amyloid beta (Aβ) peptide and intraneuronal neurofibrillary tangles consisted of paired filaments of abnormally phosphorylated tau protein (Mattson 2004; Reddy et al. 2010).

Aβ peptides are generated after sequential proteolytic cleavage of APP by two different proteases, β- and γ-secretase (Querfurth and LaFerla 2010). APP is cleaved by beta-site APP cleaving enzyme-1 (BACE1) and β-secretase to produce the secreted sAPPβ ectodomain and the membrane-bound C-terminal fragment C99. Then, C99 is cleaved by γ-secretase, which releases $A\beta_{40}$ and $A\beta_{42}$ (Selkoe 1998). $A\beta_{1-42}$ aggregates faster (Jarrett et al. 1993; Meral and Urbanc 2013), is genetically more strongly linked to AD (Sawamura et al. 2000) and forms oligomers that are more toxic (Dahlgren et al. 2002). Injection of $A\beta_{1-42}$ into hippocampal region of mouse have been widely used as an animal model of Alzheimer's disease (Lee et al. 2012; McLarnon and Ryu 2008).

Based on the "amyloid cascade hypothesis" of AD, which purports that cerebral Aβ peptide accumulation sets a neurotoxic cascade into motion (Hardy and Allsop 1991; Selkoe 2001), a great deal of focus has been directed toward anti-amyloid therapies that reduce production or enhance clearance of cerebral Aβ (Kukar et al. 2008; Schenk et al. 1999; Tan et al. 2002; Weggen et al. 2001). However, the hypothesis fails to explain how Aβ in any of its forms causes neuronal death. This is why an extension to an amyloid cascade-inflammatory hypothesis is appropriate. Chronic neuroinflammation associated with persistent glial activation is a major disease process evoked by Aβ and intimately associated with the progress of AD pathologies (Akiyama et al. 2000a; Wyss-Coray and Mucke 2002). The neuroinflammatory response could be involved in neural dysfunction, cell death and other

Chong Chen and Xiaohong Li contributed equally to this study.

C. Chen · X. Li · P. Gao · Y. Tu · M. Zhao · J. Li · S. Zhang · H. Liang ($\boxtimes$)
Institute of Traumatic Brain Injury and Neurology, Pingjin Hospital, Logistics University of Chinese People's Armed Police Forces, Tianjin 300162, China
e-mail: lianghaiqiantj@163.com

neurodegenerative changes, ultimately creating a vicious cycle. Multiple epidemiological and animal model studies present that NSAIDs, the most widely used anti-inflammatory agents, have a substantial sparing effect on AD (Aisen et al. 2003; Choi et al. 2013; Heneka et al. 2005).

Traditional Chinese medicine (TCM) has played a critical role in the promotion of health, prevention of disease, and treatment of illness for thousands of years in China and other Asian countries (Bochorakova et al. 2003; Wang et al. 2011). Baicalin (Fig. 1) is one of the main bioactive flavone glucuronides derived as a medical herb from the dried roots of Scutellaria baicalensis Georgi, and it is widely used for the treatment of fever, inflammation, and other conditions (Zhao et al. 2013). Recent studies revealed that baicalin could protect against neuronal cell death and enhance neurological function following cerebral ischemia (Cheng et al. 2013; Jung et al. 2008; Tu et al. 2009). Even though the exact mechanism was not reported, Zhang et al. (2006) demonstrated that baicalin passed through the blood–brain barrier and distributed within brain tissue, specifically in the hippocampus, striatum, cortex, and thalamus. Baicalin was also detectable in cerebral spinal fluid in the rabbit model of brain edema (Huang et al. 1999). Thus, we hypothesized that baicalin would exhibit neuroprotection against neuronal injuries.

The present study evaluated the neuroprotective effects of baicalin on AD-like pathological changes and behavioral deficits induced by an intrahippocampal injection of $A\beta_{1-42}$ protein.

Methods

Animals

50 male ICR mice (25–30 g, 6 weeks old) at the start of the experiment were used. The animals were housed in controlled room temperature (22±2 °C) and humidity (50—60 %) under a 12 h:12 h light–dark cycle (lights on 06:00 h). Accessible water and food was provided ad libitum. All procedures used
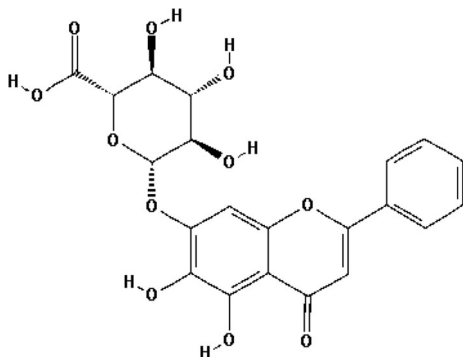

Fig. 1 Molecular structure of baicalin

in the present study followed the "National Institutes of Health Guide for Care and Use of Laboratory Animals" (Publication No. 85–23, revised 1985) and were approved by the local Animal Ethics Committee. Also, Efforts were made to minimize animal suffering and to reduce the number of animal used.

$A\beta_{1-42}$ protein injection

Human $A\beta_{1-42}$ protein (Tocris, Ellisville, MO, USA) was prepared as stock solution at a concentration of 1 mg/ml in sterile 0.1 M phosphate-buffered saline (PBS), and aliquots were stored at −20 °C. Aβ solution was aggregated by incubation at 37 °C for 4 days before use. Mice were injected with aggregated $A\beta_{1-42}$ protein into the hippocampus as described beforehand (Lee et al. 2012). In brief, mice were anesthetized with isoflurane (2.5 %). The sterilization of the injection site was carried out using gauze embedded in 70 % ethanol. A volume of 1 μl of $A\beta_{1-42}$ solution was injected into the hippocampus bilaterally at the following stereotaxic coordinates using Hamilton's microsyringe: anteroposterior, −2.00 mm from bregma; mediolateral, ±1.50 mm from midline; and dorsoventral, −1.00 mm from dura. The needle was removed after 5 min using three intermediate steps with a 1-min inter-step delay to minimize backflow. Mice were placed on a thermal pad (32–33 °C) until they awakened (Cai et al. 2011).

Drug treatment

Baicalin was purchased from Ankang Dongke Maidisen Nature Pharmaceutical Co., Ltd. (Shaanxi, P.R. China). It is suspended in Tween 80, which is a nanostructured carrier that has been demonstrated to improve oral bioavailability of baicalin and prolong its retention time in blood (Zhao et al. 2013).

50 mice were randomly divided into 5 groups; In baicalin 30 ($n=10$), baicalin 50 ($n=10$), and baicalin 100 ($n=10$) groups, baicalin was dissolved in 10 % Tween 80 and was administrated immediately after $A\beta_{1-42}$ protein injection and once daily for 14 days at a concentration of 30, 50 and 100 mg/kg body weight by gavage, respectively. The doses were based on some previous reports (Liu et al. 2013; Xue et al. 2010), which were proved to be safe in the mouse model of neurologic diseases. In control group, animals ($n=10$) were injected with $A\beta_{1-42}$ protein, and received 10 % Tween 80 solution orally. 10 sham animals were injected with the same amount of sterile saline (1 μl), and received 10 % Tween 80 solution orally.

Morris Water Maze Test

The Morris water maze procedure was used for evaluating the memory of mouse. The experimental apparatus consisted of a

circular tank (diameter=120 cm, height=50 cm) that was divided into four quadrants, filled with water and maintained at 22±2 °C. At first, a visible platform test was performed, which confirmed that there were no significant differences in sensory, motor or motivational activities among these five groups. Then, hidden platform and reverse hidden platform tests were performed in succession. For the hidden platform test, a round platform (diameter=9 cm) was placed at the midpoint of the fourth quadrant, 2 cm below the water surface. A training trial was conducted once a day for 5 days. During each trial, the mice were placed in the water at a fixed position, opposite the platform and at the edge of the pool. The mice were allowed to swim freely until they escaped onto the platform. In each trial, the swimming pathway and latency locating the hidden platform was recorded with a camera. The reverse hidden platform test was identical to the hidden platform test except the locations of the platform and the swim start were reversed; the reverse hidden platform trial lasted for 3 days.

Probe test

To assess memory consolidation, a probe test was performed 24 h after the Morris water maze test. For the probe test, the platform was removed and the mice were allowed to swim freely. The swimming pattern of every mouse was recorded with a camera. Consolidated spatial memory was estimated by the time spent in the target quadrant area.

Immunohistochemistry

Mice were deeply anesthetized with ether and sacrificed. Their brains were taken and post-fixed in PBS containing 4 % paraformaldehyde overnight at 4 °C. Post-fixed brains were cut into two hemispheres; hemispheres were embedded in paraffin, serially sectioned (3 μm) and mounted on silan-covered slides. Immunohistochemistry was carried on these sections using Iba-1 antibody (1:200; Abcam, Cambridge, UK) for activated microglia and GFAP antibody (1:200; santa cruz, Dallas, USA) for activated astrocytes. Following quenching of endogenous peroxidase with 1.5 % hydrogen peroxide in methanol (v/v) for 20 min, high temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98 °C in 10 mM trisodium citrate buffer pH 6.0, for 45 min. After overnight incubation at 4 °C with primary antibodies, the slides were washed with PBS and incubated with the appropriate biotinylated secondary antibody (Abcam, Cambridge, UK) for 50 min at room temperature. The sections were washed in PBS, and the detection was performed with horserad-ish peroxidase–streptavidin solution (Abcam, Cambridge, UK). After 30 min of incubation at room temperature, sections were washed with PBS and the detection was completed by using DAB (3,30-diaminobenzidine, Abcam, Cambridge, UK) in chromo-gen solution, followed by counter-staining with Harris's hematoxylin. Control and experimental tissues were placed on the same slide and processed under the same conditions (Passos et al. 2010).

Image analysis

After immunostaining, hemispheres sections were examined by light microscopy (Nikon, Shanghai, China). Iba-1 and GFAP immunostaining were evaluated at cross-sections of hemispheres, especially focused on hippocampus. Iba-1$^+$ and GFAP$^+$ cells in hippocampus were counted by cellular nuclear.

Western blot analysis

Western blot analysis were carried out to detect the expression of TNF-α and IL-6 in the brains. Isolated hippocampal tissues from both hemispheres were lysed briefly with protein lysis buffer (50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 1 % NP-40, 0.1 % sodium dodecyl sulfate, 1 mmol/l dithiothreitol, 0.01 g/l aprotinin, 0.01 g/l leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride) and lysed in 4 °C for 1 h. Samples of homogenates were injected to SDS-PAGE (8 % gel). Proteins were transferred to PVDF membranes in transfer buffer [25 mM Tris–HCl buffer (pH 7.4) containing 192 mM glycine and 20 % v/v methanol] at 400 mA for 2 h at 4 °C. Western blots were incubated for 3 h with a blocking solution (5 % skim milk) at room temperature followed by incubation in anti-TNF-α and anti-IL-6 antibody for 24 h at 4 °C. Then, it was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Each membrane was developed using a chemiluminescence system Fluorchem HD2 (Alpha, Innotech Corporation, Miami, FL, USA). The blots were stripped and incubated with an anti-β-actin antibody. TNF-α and IL-6 expression levels were normalized to the actin levels on the same membranes.

Statistical analysis

The distribution of all continuous variables that followed a normal Gaussian distribution are presented as mean±SEM and compared using one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test. Repeated measure analysis of variance would be conducted for evaluating the difference among groups over time in the Morris water maze test. Statistical significance was defined as P values<0.05. All statistical analyses were performed with the SPSS statistical software program package (SPSS version 15.0 for windows, SPSS Inc., Chicago, Illinois, USA).

Results

Baicalin ameliorated Aβ$_{1-42}$ protein-induced cognitive dysfunction

In the hidden platform test, the escape latency time (Fig. 2a) was dependent on both the time effect ($P<0.05$) and the group effect ($P<0.01$); the sham and baicalin 100 groups escaped significantly faster than the control group (both $P<0.05$). A similar result was observed for the swimming distance ($P<0.05$ for the time effect; $P<0.01$ for the group difference; both $P<0.01$ for the comparisons of the sham group and baicalin 100 group to the control group. Fig. 2b). Swimming speed was not significantly different among these groups (Fig. 2c).

In the reverse hidden platform test, the time effect and difference among these groups were both significant in the escape latency time ($P<0.01$ and $P<0.01$ respectively, Fig. 2d) and swimming distance ($P<0.05$ and $P<0.01$ respectively, Fig. 2e). Compared to control group, the escape latency time and swimming distance in the sham group and baicalin 100 group were

significantly shorter (all $P<0.01$). No significant differences in the swimming speed were observed (Fig. 2f).

After the Morris water maze test, we conducted a probe test to analyze maintenance of memory. During the probe test, the time spent in the target quadrant by the mice treated with baicalin 100 was significantly increased compared with control mice (Fig. 3, $P<0.01$).

However, the changes in the water maze test and in the probe test in baicalin 30 and baicalin 50 groups were not significant. Thus, we adopted 100 mg/kg of baicalin for further studies.

Baicalin attenuated Aβ$_{1-42}$ protein induced glia activation

In this present study, HE staining was used for identifying the different macroscopic pathological features in the hippocampus. Representative coronal sections from hippocampus were showed in Fig. 4. The injection of Aβ$_{1-42}$ protein increased the number of Iba-1 and GFAP positive cells. After 14 days treatment of baicalin at the concentration of 100 mg/kg, the

Fig. 2 Effects of baicalin on cognitive function evaluated with the Morris water maze test. Escape latency time, swimming distance and swimming speed from the hidden platform test (a, b and c, respectively) and the reverse hidden platform test (d, e and f, respectively) were shown. *$P<0.05$ compared to control group
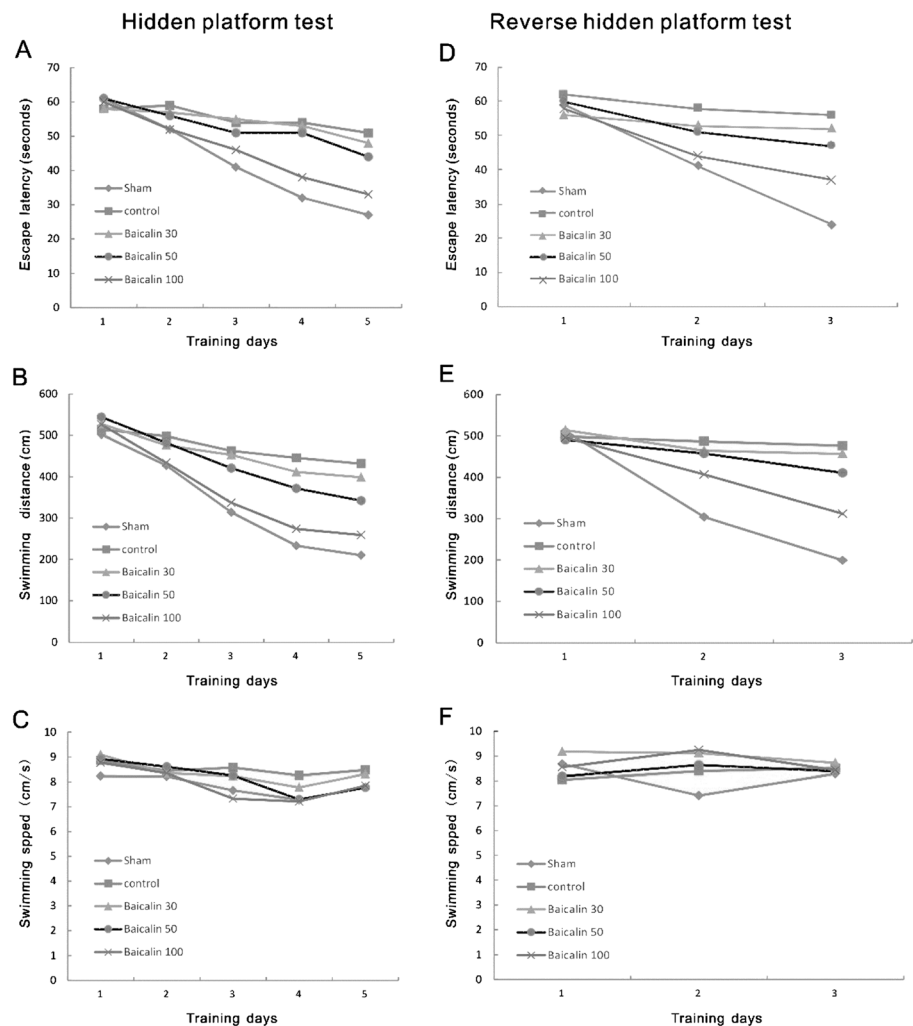
number of Iba-1$^+$ microglia was reduced, compared to control group ($P<0.01$, Fig. 4d). Similar results were observed in astrocytes, which were detected by an anti-GFAP antibody. It is obvious that administration of 100 mg/kg of baicalin significantly attenuated astrocyte activation ($P<0.01$, Fig. 4h).

Baicalin administration reduced Aβ$_{1-42}$ protein induced proinflammatory cytokines expression

To further identify the effect of baicalin on hippocampal neuroinflammation involved in AD, the expression of pro-inflammatory cytokines were examined. It has been demonstrated that the expression pro-inflammatory cytokines would increase significantly in the amyloid β$_{1-42}$-induced mice. Our results showed that TNF-α ($P<0.01$, Fig. 5a) and IL-6 ($P<0.05$, Fig. 5b) were significantly reduced in baicalin 100 group, compared to control group.

Discussion

The inflammatory reaction induced by Aβ involves the release of damaging factors such as TNF-α and IL-6, which promote activation of intracellular pathways that contribute to the progression of AD (Walsh and Selkoe 2004). The development of therapies for this neurodegenerative disorder represents a major challenge to academic, biotechnology, and pharmaceutical researchers. Epidemiological data demonstrated that flavonoids might reduce the risk rate for the incidence of AD. Meanwhile, the anti-inflammatory ability of the flavonoid family have been considered to provide rational for this hypothesis (Letenneur et al. 2007). Baicalin, a flavone derived from the dried roots of Scutellaria baicalensis Georgi, has multiple effects such as antioxidant, anticancer and anti-

Fig. 3 Effect of baicalin on improvement of memory impairment evaluated with the probe test. 24 h after the Morris water maze, a probe test was performed and the time spent in the target quadrant by the mice treated with 100 mg/kg of baicalin was significantly increased. *$P<0.05$ compared to control group

inflammatory effects (Ikemoto et al. 2000; Li et al. 2000; Shieh et al. 2000). Moreover, recent studies revealed that baicalin possessed neuroprotective effects in the ischemic cerebral injury (Xue et al. 2010; Zhou et al. 2013), which raised the possibility of using baicalin as a potential agent for neurodegenerative disorders such as AD. Our results firstly demonstrated that 100 mg/kg of baicalin treatment might ameliorated memory impairment, and also attenuated glial cell activations and increase of TNF-α and IL-6 expressions induced by Aβ$_{1-42}$ protein.

Previous studies have demonstrated that Aβ peptide administrated induced behavioural changes specific to learning and memory (Colaianna et al. 2010; Passos et al. 2010). In the hidden platform and reverse platform tests, the escape latency time and swim distance both significantly decreased as the training proceeded, indicating an ongoing spatial learning and memory in the mice; however, the extent of the decreases in the baicalin 100 group was significantly higher in comparison to control group. There was no significant difference in the swimming speed among these groups, which indicates that 100 mg/kg of baicalin might attenuate the Aβ$_{1-42}$ induced impairment in spatial learning and memory. In the probe test, the time spent in the target quadrant was also significantly increased in the mice treated with 100 mg/kg of baicalin. Hippocampus is one of the pivotal regions of learning and memory, especially temporary memory, and its injury is recognized as a key procedure for AD (Reilly et al. 2003). Thus, the effects of 100 mg/kg of baicalin on the pathological changes of hippocampus would be examined.

Previous studies demonstrated that increased inflammatory components contributed to the progression and acceleration of AD by activating plaque-associated microglia and astrocytes. It is now well established that most types of injury or pathological process within the CNS lead to activation of those glia from their resting state (Gao and Hong 2008; Kreutzberg 1996). Microglial and astrocyte activation have been previously demonstrated in the brains of AD patients (Akiyama et al. 2000b). Therefore, the inhibition of microglia and astrocyte is a therapeutic target for the treatment of neurodegenerative disorders. In this study, the numbers of Iba-1 positive microglia and GFAP positive astrocytes were significantly decreased by 100 mg/kg of baicalin administration, indicating that baicalin attenuated Aβ$_{1-42}$ protein-induced activation of microglia and astrocytes in hippocampus.

In general, under physiological conditions, pro-inflammatory cytokines such as TNF-α and IL-6 are absent or expressed at very low level in the brain. However, they can be induced by amyloid peptide in astrocytes, microglia, neurons and endothelial cells, leading to neurodegeneration. The inflammation is mediated by pro-inflammatory cytokines and would create a chronic and self-sustaining inflammatory interaction between activated microglia and astrocytes, stressed neurons and Aβ plaques (Rubio-Perez and Morillas-Ruiz 2012). In the present study, we
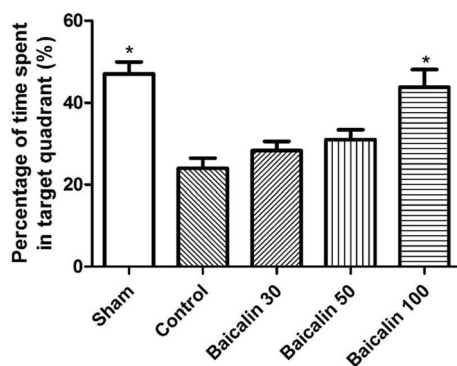
Fig. 4 Effects of baicalin on pathological changes in the hippocampus of mice after $A\beta_{1-42}$ injection. **a-c** photomicrographs of Iba-1 positive cells in the hippocampus of sham, control and baicalin 100 group; **d** Numbers of Iba-1 positive microglia in sham and baicalin 100 groups were lower than that in control group; **e-g** GFAP staining in the hippocampus of sham, control and baicalin 100 group; **h** The bar charts showed that the number of GFAP positive astrocytes in the sham and baicalin 100 groups was lower than that in control group. *$P<0.05$ compared to control group
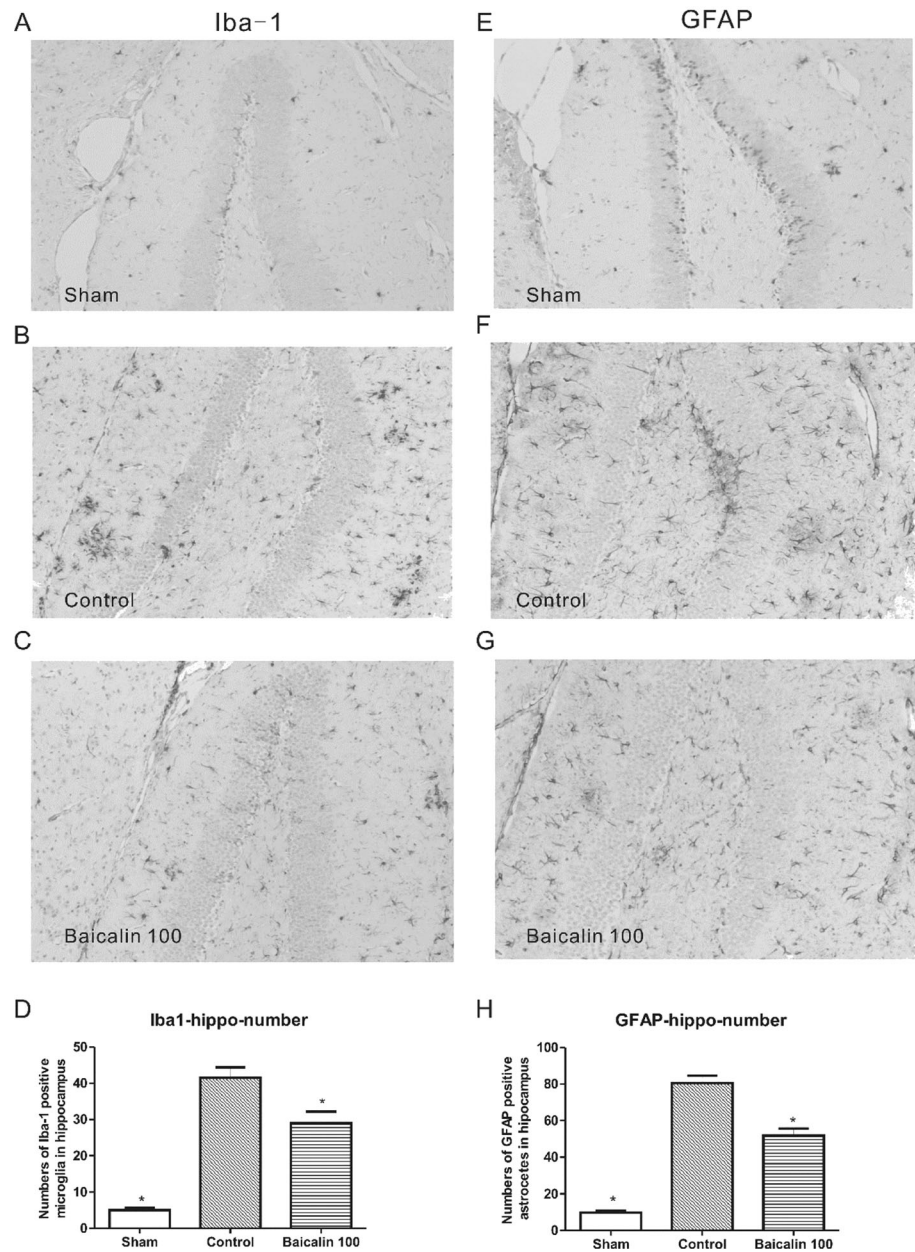
found that 100 mg/kg of baicalin statistically reduced the intracellular expressions of TNF-α and IL-6 in the hippocampus of the mouse brain.

In conclusion, our results demonstrated that 100 mg/kg of baicalin restored $A\beta_{1-42}$ protein-induced cognitive function, attenuated the activation of microglia and astrocytes, and
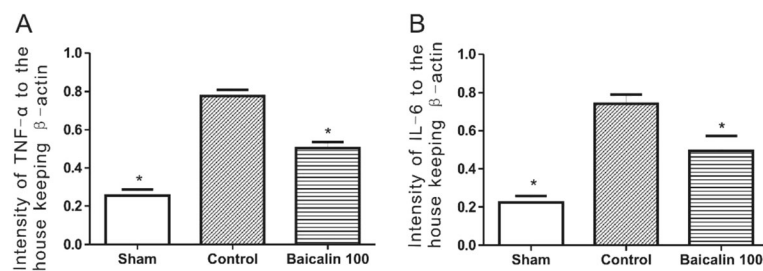

Fig. 5 Effects of baicalin on the expressions of pro-inflammatory cytokines. The bar charts showed that TNF-α **a** and IL-6 **b** levels in the sham and baicalin 100 groups were significantly decreased, compared to control group. *$P<0.05$ compared to control group

reduced the expression of proinflammatory cytokines, suggesting that baicalin might be a potential therapeutic option of AD and related neurodegeneration. However, as the mouse model we used can not fully represent human AD pathology and some additional elements such as diet, social as well as environmental factors is difficult to mimic in animals, the bioavailability extend of baicalin is still need to be evaluated in further research.

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