Original paper

Folia Neuropathologica

Protective effects of fisetin in an Aβ_{1-42}-induced rat model of Alzheimer's disease

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Folia Neuropathol 2023; 61 (2): 196-208 *DOI: https://doi.org/10.5114/fn.2023.126893*

Abstract

Alzheimer's disease (AD) is a chronic, neurodegenerative disorder that affects the central nervous system and is found predominantly in elderly populations. As amyloid β protein (Aβ) is one of the key players responsible for the pathogenesis of AD, we sought to investigate the protective effects of fisetin in an Aβ_{1-42}-induced rat model of AD. In this model, the protective effects of fisetin on learning and memory impairment induced by Aβ_{1-42} were determined via the Morris water maze and passive avoidance test. Furthermore, the antioxidant activity, anti-inflammation, and apoptosis effect of fisetin were investigated using biochemical and immunohistochemical methods. The results showed that intragastric (i.g.) administration of fisetin (100, 50, and 25 mg/kg) improved previous learning and memory impairments in Aβ_{1-42}-treated rats. Hippocampal tissue from these fisetin-treated rats revealed that the activities of total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px) were markedly enhanced, and that the levels of malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were significantly reduced. Meanwhile, fisetin also significantly attenuated Aβ_{1-42}-induced cholinergic dysfunction such as elevated the activity of choline acetyltransferase (ChAT) and reduced the activity of acetylcholine esterase (AChE). In addition, hippocampal tissue obtained from fisetin-treated rats revealed a reversal of Aβ_{1-42}-induced effects on apoptotic pathway protein (caspase-3) expression and inflammatory response of glial fibrillary acidic protein (GFAP). This indicated that the amount of degenerating hippocampal neurons with apoptotic features was dramatically reduced after treatment with fisetin. Collectively, these findings suggest that fisetin has potential as a treatment agent for Alzheimer's disease and that its effects occur through several mechanisms, including inhibition of oxidative stress, adjustments to previous cholinergic dysfunction, anti-inflammatory actions, and decreased apoptotic activity.

Key words: *Alzheimer's disease, Aβ_{1-42}, fisetin, protective effects, mechanism.*

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease associated with aging. Hallmark clinical features of AD include memory loss, cognitive dysfunction, and abnormal changes to the individual's personality – all of which are hallmark features of chronic central nervous system degeneration [39]. According to the data released by the World Alzheimer's Disease Report 2022, there are currently 6.5 million AD patients over the age of 65 in the United States. This burden is hypothesized to increase dramatically, with the number of AD patients reaching 13.8 million by 2060 [1]. With the accelerated aging of the world, AD has seriously affected the quality of life and health of the elderly. As such, treatment and prevention of AD have become an urgent and difficult problem to be solved.

The typical pathological changes of AD are the appearance of extracellular deposits of β-amyloid (Aβ) generated senile plaques (SP) [34], intracellular neuro-

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fibrillary tangles [2], cholinergic loss [9], and neuron loss in key brain areas of memory and cognition. Increasing evidence suggests that brain accumulation of Aβ is the crucial link between the pathological changes found in AD and can result in a series of pathological processes [8]. Excessive deposition of Aβ can cause excitatory neurotoxicity and neuronal apoptosis, which will further aggravate the pathological development of AD [16]. To this end, this method has been approved by researchers that $A\beta_{1-42}$ oligomers injected into the hippocampus of the rat, caused a series of neurotoxic effects, induced dysfunction and even death of nerve cells, resulting in decreased memory ability, resulting in an AD model [28]. Moreover, Aβ deposition can induce chronic inflammation and oxidative stress that results in the formation of reactive oxygen species (ROS) [4]. During conditions of oxidative stress, there is a serious imbalance between the body's oxidation and antioxidant systems, and this imbalance ultimately leads to lipid peroxidation, protein carbonyl formation, and DNA damage such as the oxidation of deoxyguanosine to form 8-hydroxy-2'-deoxyguanosine (8-OHdG) [10].

Although there is an abundance of research investigating the mechanism(s) behind AD, we still do not have an effective way to prevent or to cure this disease. Given this, we sought to combine our country's rich natural resources and explore candidate AD substances that have good therapeutic potential, but low side effects. It is hoped that this approach will play a positive role in the future treatment and prevention of AD.

Fisetin is a dietary flavonoid 3,7,3',4'-tetrahydroxyflavone (see Fig. 1 for detailed structure), that is a compound extracted from natural plants [15]. It has been described as a potent natural molecule with multiple beneficial biological activities, including: anti-oxidation [21], anti-inflammatory [6], anticancer [33], neuroprotection [36,37], postponement of senility, enhanced memory [35]. In this study, we sought to investigate the protective effects of fisetin on learning and memory impairment in an $A\beta_{1-42}$-induced rat model of AD. We then explored its underlying mechanism, focusing on its effects on oxidative damage, cholinergic dysfunction, inflammatory response, and neuronal apoptosis.

Material and methods

Material

Fisetin was purchased from Xi'an Jinnking Biological Technology Co., LTD (Xian, China). $A\beta_{1-42}$ was obtained from GL Biochem Ltd (Shanghai, China). Assay kits for malondialdehyde (MDA), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), choline acetyltransferase (ChAT), and acetylcholine esterase (AchE) were acquired from Jiancheng Bioengineering Institute (Nanjing, China). The DNA fragmentation detection kit (TUNEL) was obtained from Roche Molecular Biochemicals (Germany). 3,3'-diaminobenzidine (DAB) substrate kit was purchased from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing China). All other common chemicals and reagents were of analytical grade.

Preparation of aggregated $A\beta_{1-42}$

Soluble $A\beta_{1-42}$ oligomers were prepared based on a previously published process [22,25]. $A\beta_{1-42}$ was dissolved into hexafluoroisopropanol (HFIP, Sigma) to achieve a 1 mM concentration, incubated until the solution was clear and transparent, and vacuum dried to remove residual HFIP. Dimethyl sulfoxide (DMSO) was then added to obtain a 5 mM solution, after which phosphate-buffered saline (PBS, pH 7.4) was added to yield a final concentration of 2.5 μg/μl. The solution was incubated at 4°C for 24 h and soluble $A\beta_{1-42}$ oligomers were stored at –80°C for later use.

Animal groups and drug delivery

Seventy-two adult male Sprague-Dawley (SD) rats (Weitong Lihua Experimental Animal Central; User License: SCXK (jing) 2012-0001) were used, all of which weighed 180-200 g and were approximately six weeks old. All animals were provided with *ad libitum* access to food and water and maintained in a temperature- (22 ±1°C) and humidity- (55 ±5%) controlled room under a 12-h light-dark cycle (7 am-7 pm). Rats were randomly assigned to one of the six following groups (n = 12 animals per group): sham control group (0.01 M phosphate-buffered saline [PBS] + sterile water), Model group ($A\beta_{1-42}$ + sterile water), Huperzine A group ($A\beta_{1-42}$ + 0.2 mg/kg huperzine A) as a positive control group, Fisetin-H group ($A\beta_{1-42}$ + 100 mg/kg fisetin), Fisetin-M group ($A\beta_{1-42}$ + 50 mg/kg fisetin), and Fisetin-L group ($A\beta_{1-42}$ + 25 mg/kg fisetin).

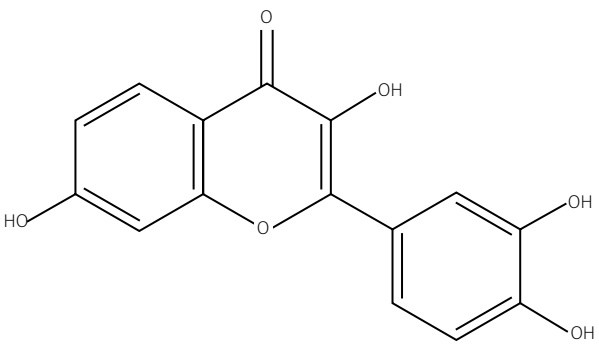

Fig. 1. Structure of the flavonoid fisetin.

Surgery and administration

Rats were anesthetized using an intraperitoneal (i.p.) injection of 2% sodium pentobarbital (0.35 ml/100 g) and securely affixed to the stereotaxic apparatus. A longitudinal incision was made through the skin overlaying the skull and the subcutaneous tissue was separated. Stereotaxic coordinates for the rat hippocampal CA1 region were determined using the Paxinos' and Watson's atlas of the rat brain [20], which were as follows: anterior-posterior (3.3 mm), medial-lateral (±2.0 mm), and dorsal-ventral (3.4 mm). Holes were bored above these axis coordinates and 5 µl of incubated $A\beta_{1-42}$ (2.5 µg/µl) was injected bilaterally over 10 min using a Hamilton microsyringe. The syringe was kept in place for an additional 5 min to ensure that the solution had fully diffused from the injection site. After injection, each rat was sutured and injected with 80000 U penicillin to prevent post-op infection. Both the sham and model groups were injected bilaterally with an equivalent volume of phosphate buffer saline (0.01 M PBS). The first day after surgery, fisetin (25, 50, or 100 mg/kg) or huperzine A (0.2 mg/kg, positive control) groups were given their respective drug doses by intragastric administration once daily for the following 15 consecutive days. Sham and model groups received the same volume of sterile water over the same injection schedule as that of the fisetin-treated groups. All animal treatment and maintenance procedures were approved by the Research Institute of Science and Technology of Functional Food and the Beijing Key Laboratory of Bioactive Substances and Functional Food, Beijing Union University.

Morris water maze test

The Morris water maze test is mainly used as a measure of spatial learning and memory [30]. Technically, it was composed of the water maze device itself, an automatic water maze image information acquisition system, and a corresponding software analysis system. The tank (145 cm in diameter and 60 cm high) was enclosed with a black cloth curtain and tank wall and water were all black. This was to prevent the presence of any visual cues. On experimental days, the water temperature was kept at 24°C ±1°C. The tank was randomly divided into four quadrants (northeast, southeast, southwest, and northwest) and an escape platform was placed in the centre of any of these quadrants, at a depth of 2 cm below the surface. A camera was placed above the centre of the tank to image swimming behaviour and to collect signals through the computer input. The key feature of the Morris water maze test was the inclusion of the hidden platform. This allowed for a spatial probe test to be conducted, which recorded the escape latency of each subject (or, the time it took for each subject to find the hidden platform). Thus, the spatial and directional learning abilities of the rat were assessed. The platform was then removed for the spatial probe test, which was an assessment of the rat's memory as to the location of the original platform.

The Morris water maze test began on day 11 after Aβ injection as previously described [31]. All experimental rats completed the hidden platform test and their respective escape latencies were recorded. Three daily training trials were conducted for four consecutive days. On each test, the rat was placed in the tank at one starting position and allowed to swim freely until it found the platform. Rats that found the platform were allowed to remain on the platform for 15 s and were then returned to the home cage. If a rat failed to reach the platform within 90 s, it was gently guided to the platform by the experimenter, where it remained for 15 s. The platform was removed on day 15 for the spatial probe test. The results from this test were used to evaluate the effect of fisetin on learning and memory impairment in the $A\beta_{1-42}$-induced rat model of AD.

Shuttle-box passive avoidance test

The Shuttle-box passive avoidance apparatus consisted of two compartments: a light and dark side, both of which were separated by a guillotine door. The shuttle-box passive avoidance test began on day 13 after Aβ injection. Briefly, the rat was oriented toward the chamber wall of the light room. The door separating the two rooms was opened and the rat was allowed 3 min of free activity in both the light and dark rooms. On day 14 of training, the rat was oriented toward the chamber wall in the light room. When all four limbs of the rat were completely in the dark room, the door separating the two rooms was immediately closed and the rat received an electrical shock (2 s continuously). On day 15 (24 h after training), the rat was again placed in the light room, but with no plantar electrical stimulation. The rat was observed for time it took it to enter the dark room (incubation period; within 5 min) and the number of errors entries into the dark room. If the rat failed to enter the dark room, the incubation period was denoted as 300 s.

Tissue processing

After behavioural testing, rats (n = 8 in each group) were decapitated. Brains were immediately removed and washed in ice-cold, isotonic saline solution. Hippocampal tissue was isolated and frozen in liquid nitrogen prior to analysis. The remaining rats in each group were anesthetized with sodium pentobarbital and transcardially perfused with 0.1 M PBS followed by 4% parafor-

maldehyde (pH 7.4). The brain was then removed, kept in its entirety, and placed in 4% paraformaldehyde. After embedding it in a paraffin wax block, 5 µm coronal sections were cut using a paraffin wax slicer.

Measurement of the activities of GSH-Px, T-SOD, ChAT, AChE, and the level of MDA

The activities of glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD), choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and the level of malondialdehyde (MDA) were measured using commercially available assay kits, which were purchased from Jiancheng Bioengineering Institute (Nanjing, China). All kits were used in accordance with the manufacturer's instructions.

Haematoxylin and eosin staining

Five-micron thick sections were obtained from the paraffin-embedded brain tissue. Haematoxylin and eosin (H&E) staining was performed according to a standard procedure. An automated H&E staining machine was used to observe the morphology of pyramidal neurons in the CA1 region of the hippocampus.

TUNEL assay

The TUNEL assay is able to detect DNA strand breaks that are predominantly induced by ROS and/or apoptosis [24]. The amount of DNA fragmentation was determined in our samples using a commercially available TUNEL assay kit (Roche TUNEL Kit, Roche Molecular Biochemicals, Germany) and in accordance with the manufacturer's instructions. Briefly, hippocampal slices were rinsed twice for 5 min each using dimethyl benzene and subjected to a series of three minute ethanol washes of decreasing concentrations (100%, 95%, 85%, and 75%). Tissue was then washed three times for 5 min each using PBS. Proteinase K (20 µg/ml) was then added and incubated at 37°C for 20 minutes, after which tissue was again washed three times (5 min/wash) with PBS. Samples were incubated with 3% H_2O_2 in methanol for 10 min at room temperature and rinsed in PBS twice for 5 min each. Slices were then transferred to a buffer containing TdT and fluorescein-dUTP and incubated in a humid chamber for 1 hour at 37°C. After a final series of three PBS washes (5 min/wash), samples were examined using standard light microscopy.

Immunohistochemical analysis of 8-OHdG, GFAP, and caspase-3

Aβ-induced oxidative stress conditions result in DNA damage and an association between 8-OHdG and DNA fragmentation [32]. The expression of both glial fibrillary acidic protein (GFAP) and caspase-3 have been used as neuroinflammatory and neuronal apoptosis markers in the hippocampus of AD, respectively [11,13]. Given this, 8-OHdG, GFAP, and caspase-3 were analysed using an immunohistochemical approach. Tissue sections were washed twice in xylene for paraffin removal and were then immersed in graded ethanol solutions (100%, 100%, 95%, and 75%). Sections were washed three times for 5 min each using PBS before being placed in an EDTA buffer solution (pH 9.0) and boiled in a pressure-cooker. Tissue sections were then incubated for 15 min in 3% H_2O_2 solution to remove endogenous peroxidase activity, followed by three rinses in PBS (5 min/rinse). The sections were blocked in goat serum for 20 min at 37°C (SP Immunohistochemical Staining Kit, Beijing Zhongshan Biotechnology Co., China) and incubated overnight at 4°C with one of the following: rabbit anti-8-OHdG, anti-GFAP, and anti-caspase-3 antibodies. After incubation, tissue sections were rinsed in PBS, incubated with biotinylated goat anti-rabbit IgG (SP immunohistochemical staining kit, Beijing Zhongshan Biotechnology Co., China) for 20 min at 37°C, and rinsed again. Tissue was then incubated in avidin-biotin horseradish peroxidase macromolecular complex (SP Immunohistochemical Staining Kit, Beijing Zhongshan Biotechnology Co., China) for 20 min at 37°C. Sections were incubated briefly in DAB using a DAB substrate kit and then rinsed three times in PBS. Finally, slices were dehydrated, cleared, and cover slipped with mounting medium.

Statistical analysis

All data were expressed as mean ±SD. The Morris water maze data were analysed using a two-way analysis of variance (two-way ANOVA). When group variances were heterogeneous, we used a one-way ANOVA. Statistical significance was set at $p < 0.05$.

Results

Effect of fisetin on escape latency in the Morris water maze

We used the Morris water maze to determine if fisetin could ameliorate the learning and memory deficits seen in an $A\beta_{1-42}$-induced rat model of AD. Beginning on day 11 after surgery, each group ($n = 12$) was trained in the Morris water maze for four consecutive days. With the increase of training times, the escape latencies were decreased gradually across days (Fig. 2A). As shown, rats in the model group had the worst learning performance in the Morris water maze when compared to sham controls ($p < 0.01$). Critically, rats treat-

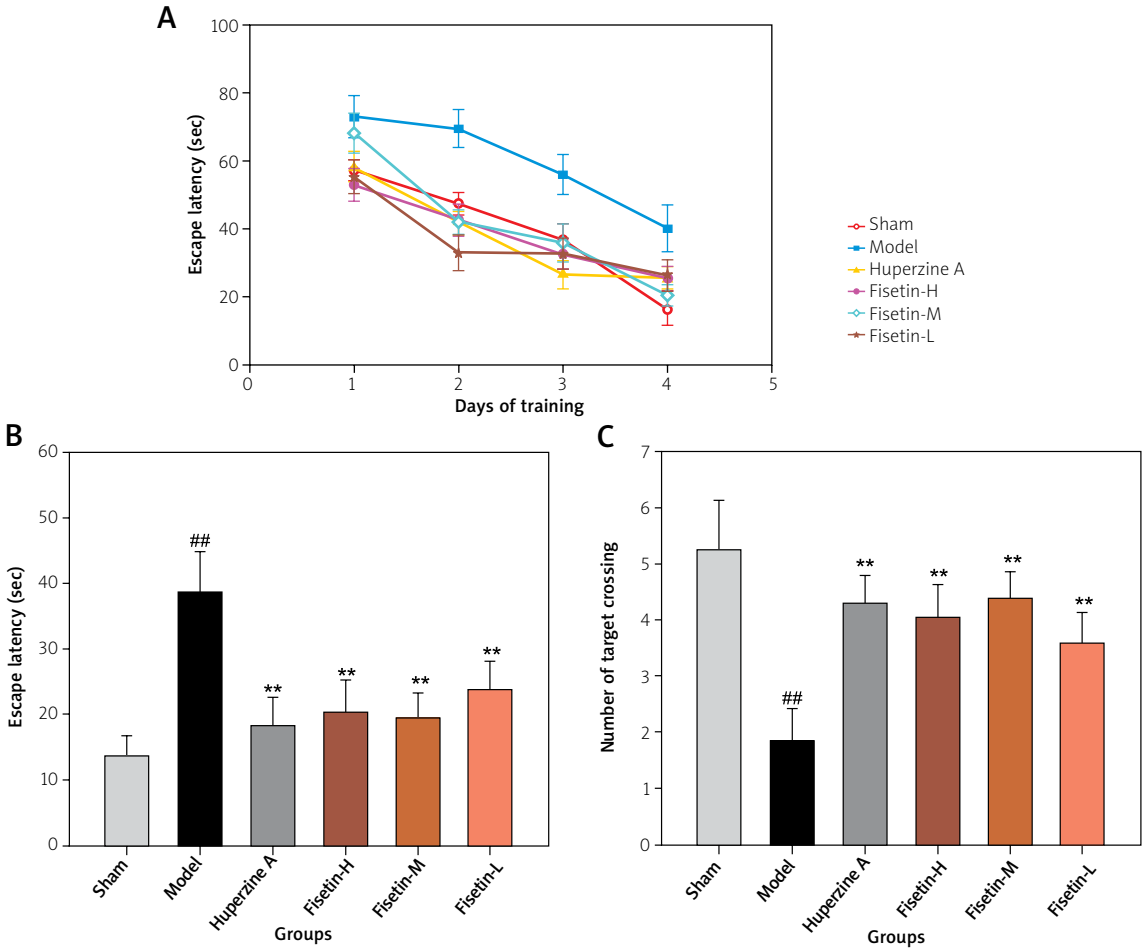

Fig. 2. Effects of fisetin on escape latency of the Morris water maze in $A\beta_{1-42}$-induced memory impairment rats. **A**) The escape latency of each training session beginning on day 11 after Aβ injection. Three training trials were administered each day for four consecutive days. **B**) The escape latency was defined as the first time the rat crossed the position in which the platform had been prior to the probe trial. **C**) The search frequency was the number of times the rat crossed the target area. Data are expressed as mean ±SD (n = 12 in each group; $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ relative to the sham group).

ed with fisetin (50 and 25 mg/kg) were able to locate the hidden platform more efficiently than those of the model group ($p < 0.05$).

As shown in Fig. 2B, the model group of $A\beta_{1-42}$-injected rats had significantly longer escape latencies in the probe test than those of the sham group ($p < 0.01$). When compared to the model group, the Huperzine A, Fisetin-H, Fisetin-M, and Fisetin-L groups (0.2 mg/kg, 100 mg/kg, 50 mg/kg, and 25 mg/kg, respectively) had markedly shorter escape latencies ($p < 0.01$), indicating improvements to $A\beta_{1-42}$-induced learning and memory deficiencies. As shown in Figure 2C, the $A\beta_{1-42}$-injected rat model group also demonstrated significantly shorter search frequency during the probe test than that of the sham group ($p < 0.01$). The number of target crossings in the Huperzine A, Fisetin-H, Fisetin-M, and Fisetin-L groups were significantly higher when compared to the model group ($p < 0.01$). We did not find any dose-dependent effects amongst the different fisetin treatment groups.

Effect of fisetin on the shuttle-box passive avoidance test

The shuttle-box passive avoidance test was traditionally known to assess learning and memory ability. As shown in Table I and when compared to the sham group, the model group's latency was significantly reduced ($p < 0.05$) while the numbers of errors was sig-

Table I. Effect of fisetin on the shuttle-box passive avoidance test

Group	Dose (mg/kg)	Incubation period	Number of errors
Sham	–	300.00 ±0.00	0.00 ±0.00
Model	–	160.08 ±122.91$^{\#}$	0.66 ±0.49$^{\#\#}$
Huperzine A	0.2	298.83 ±4.04*	0.08 ±0.29*
Fisetin-H	100	296.17 ±13.28*	0.08 ±0.29*
Fisetin-M	50	288.25 ±30.94	0.16 ±0.38
Fisetin-L	25	269.16 ±69.86	0.25 ±0.45

**p < 0.05 relative to the model group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 relative to the sham group.*

Table II. Effects of fisetin on the activities of choline acetyltransferase (ChAT) and acetylcholine esterase (AchE)

Group	Dose (mg/kg)	ChAT (U/g wet tissue)	AChE (U/mg protein)
Sham	–	84.43 ±7.79	0.18 ±0.03
Model	–	54.41 ±9.94$^{\#\#}$	0.30 ±0.06$^{\#\#}$
Huperzine A	0.2	83.06 ±6.50**	0.21 ±0.05**
Fisetin-H	100	74.79 ±9.51**	0.19 ±0.02**
Fisetin-M	50	74.10 ±6.25**	0.20 ±0.06**
Fisetin-L	25	63.50 ±4.64*	0.26 ±0.034

**p < 0.05, **p < 0.01 relative to the model group, $^{\#\#}$p < 0.01 relative to the sham group.*

Table III. Effects of fisetin on the activities of GSH-Px and T-SOD, and the level of MDA

Group	Dose (mg/kg)	GSH-Px (U/mg protein)	T-SOD (U/mg protein)	MDA (nmol/mg protein)
Sham	–	25.20 ±6.06	133.11 ±7.05	3.20 ±0.59
Model	–	18.32 ±2.39$^{\#\#}$	88.18 ±6.06$^{\#\#}$	9.14 ±3.02$^{\#\#}$
Huperzine A	0.2	23.88 ±5.04*	130.70 ±8.67**	3.43 ±0.87**
Fisetin-H	100	19.45 ±1.97	131.53 ±5.23**	2.86 ±0.68**
Fisetin-M	50	24.01 ±7.74*	128.85 ±4.31**	4.35 ±1.47*
Fisetin-L	25	24.18 ±3.77*	113.26 ±10.98**	4.44 ±2.19*

**p < 0.05, **p < 0.01 relative to the model group, $^{\#\#}$p < 0.01 relative to the sham group.*

nificantly increased ($p < 0.01$). When compared to the model group, 14 days of treatment with either Huperzine A or Fisetin-H (100 mg/kg) significantly increased escape latency ($p < 0.05$) and reduced the numbers of errors ($p < 0.05$) in the passive avoidance test.

Effects of fisetin on the activities of ChAT and AChE

Alzheimer's disease was characterized by marked cholinergic dysfunction [18]. As shown in Table II, $A\beta_{1-42}$ administration to rats resulted in altered activities of ChAT and AChE in the hippocampus. When compared to the sham group, the activity of ChAT significantly decreased ($p < 0.01$), while the activity of AChE significantly increased in the $A\beta_{1-42}$-induced model group. Relative to the model group, the activity of ChAT in Huperzine A, Fisetin-H, Fisetin-M, and Fisetin-L treatment groups were markedly enhanced ($p < 0.01$, $p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively). Moreover, the activity of AChE in Huperzine A, Fisetin-H, and Fisetin-M treatment groups were all significantly reduced ($p < 0.01$). Fisetin-L treatment had no significant effect on AChE activity.

Effects of fisetin on the activities of GSH-Px and T-SOD, and MDA levels

As oxidative stress plays an important role in $A\beta_{1-42}$-induced neurotoxicity [12], the effects of fisetin treatment on the activities of GSH-Px and T-SOD, and the level of MDA in the hippocampus were next investigated. The results showed that $A\beta_{1-42}$ treatment in the model group resulted in significantly ($p < 0.01$) decreased activities of both GSH-Px and T-SOD, compared to the sham group (Table III). In addition, the level of MDA was significantly increased in model group compared with shame group ($p < 0.01$). However, relative to the model group, the activities of the antioxidant enzymes GSH-Px in the Huperzine A, Fisetin-M (50 mg/kg), and Fisetin-L (25 mg/kg) treatment groups were significantly increased ($p < 0.05$). Rela-

tive to the model group, the activities of the antioxidant enzymes T-SOD in the Huperzine A, Fisetin-H (100 mg/kg), Fisetin-M (50 mg/kg), and Fisetin-L (25 mg/kg) treatment groups were significantly elevated ($p < 0.01$). Finally, relative to the model group, MDA levels were significantly reduced in groups receiving pretreatment with Huperzine A, Fisetin-H, Fisetin-M, or Fisetin-L ($p < 0.01$, $p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively).

Fisetin suppressed 8-OHdG levels

8-OHdG was a sensitive marker of DNA damage [19]. Immunohistochemical data revealed that the levels of 8-OHdG in the CA1 region of the hippocampus were significantly enhanced in the AD model group ($p < 0.01$). Interestingly, this increase was suppressed by treatment with Huperzine A, Fisetin-H, Fisetin-M, and Fisetin-L (Fig. 3A and B, all $p < 0.01$).

Effects of fisetin on hippocampal neuronal morphology

H&E staining was used to investigate the effects of fisetin on $A\beta_{1\text{-}42}$-induced neuronal morphological change in the CA1 region of the hippocampus. Representative images for this H&E staining from each group is shown in Figure 4. The results indicated that pyra-

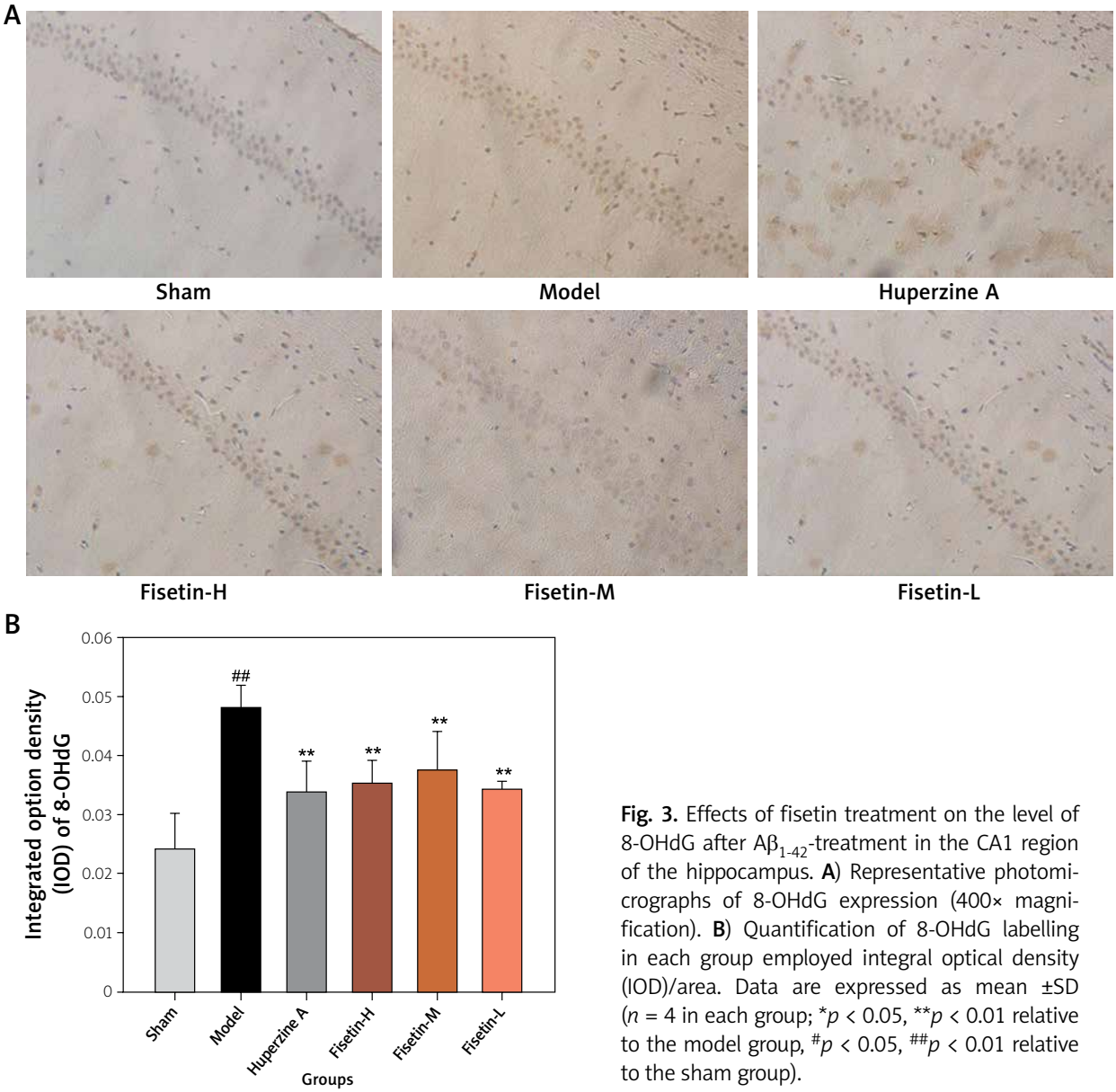

Fig. 3. Effects of fisetin treatment on the level of 8-OHdG after $A\beta_{1\text{-}42}$-treatment in the CA1 region of the hippocampus. **A**) Representative photomicrographs of 8-OHdG expression (400× magnification). **B**) Quantification of 8-OHdG labelling in each group employed integral optical density (IOD)/area. Data are expressed as mean ±SD ($n = 4$ in each group; $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ relative to the sham group).

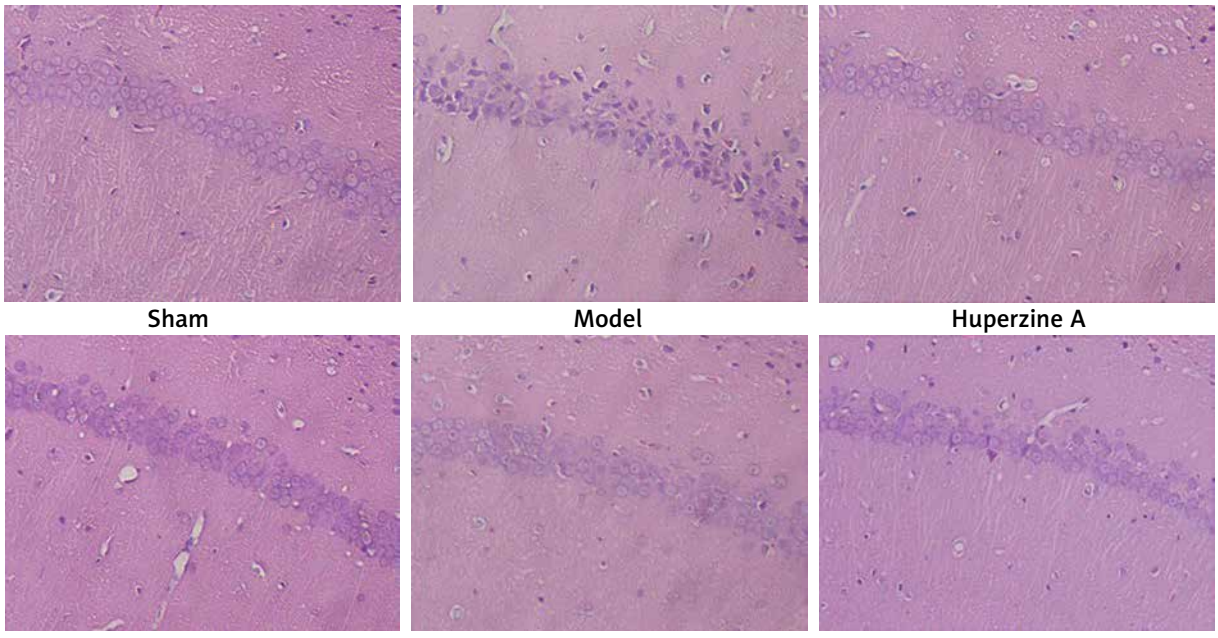

Fig. 4. Effects of fisetin on hippocampal neuronal morphology. H&E histochemical staining in the CA1 region of the hippocampus for each group. Representative photomicrographs of H&E labelling in hippocampus (400× magnification; $n = 4$ in each group).

midal cells of the CA1 region obtained from the sham group were closely packed with clear nuclear staining. By contrast, the pyramidal cells of the $A\beta_{1-42}$-induced AD model group had significant damage, revealed in nucleoli ambiguity, indefinable borders between nuclei and cytoplasm, non-structurally intact pyramidal cells, and decreased amounts of morphologically healthy neurons. Interestingly, neurons from fisetin-treated groups had markedly reversed $A\beta_{1-42}$-induced CA1 neuronal damage, maintaining their pathological lesions close to normal, control ranges. This was particularly evident for doses of 50 and 100 mg/kg.

Effect of fisetin on $A\beta_{1-42}$-induced hippocampal neuronal apoptosis

The TUNEL assay was used to investigate the effects of fisetin on $A\beta_{1-42}$-induced hippocampal neuronal apoptosis in the area surrounding the damaged site (Fig. 5). The data provided supporting evidence that there was a significant increase in the apoptotic neurons of rats in the model group when compared with those in the sham group ($p < 0.01$, Fig. 5). However, fisetin treatment at doses of 25, 50 and 100 mg/kg markedly decreased the number of apoptotic cells ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively). Apoptotic cells were occasionally observed in the Huperzine A treatment group ($p < 0.01$, Fig. 5).

Fisetin reduced GFAP and caspase-3 protein levels

Glial fibrillary acidic protein was primarily expressed in astrocytes that are involved in inflammatory reactions and was used by us as a marker of astrogliosis [11]. Figure 6 shows the effects of fisetin on GFAP levels in the CA1 region of the hippocampus. Fisetin significantly increased both the area and intensity of GFAP staining in the AD model group. However, this increase was largely reversed in all fisetin treatment groups.

Furthermore, our immunohistochemical data also examined the expression of caspase-3 in the CA1 region of the hippocampus. When compared with the sham group, caspase-3 levels were significantly enhanced in the AD model group ($p < 0.01$). Fisetin treatment (25, 50 and 100 mg/kg) and huperzine A treatment all significantly decreased the expression of caspase-3, when compared to the model group (Fig. 7A and B, $p < 0.01$).

Discussion

Alzheimer's disease is a type of brain dysfunction featuring a gradual loss in memory and its pathological features. Aβ deposition is believed to occupy an important role in the pathogenesis and progression of AD [23]. Aβ deposition in the brain can generate a range of negative responses, including oxidative damage,

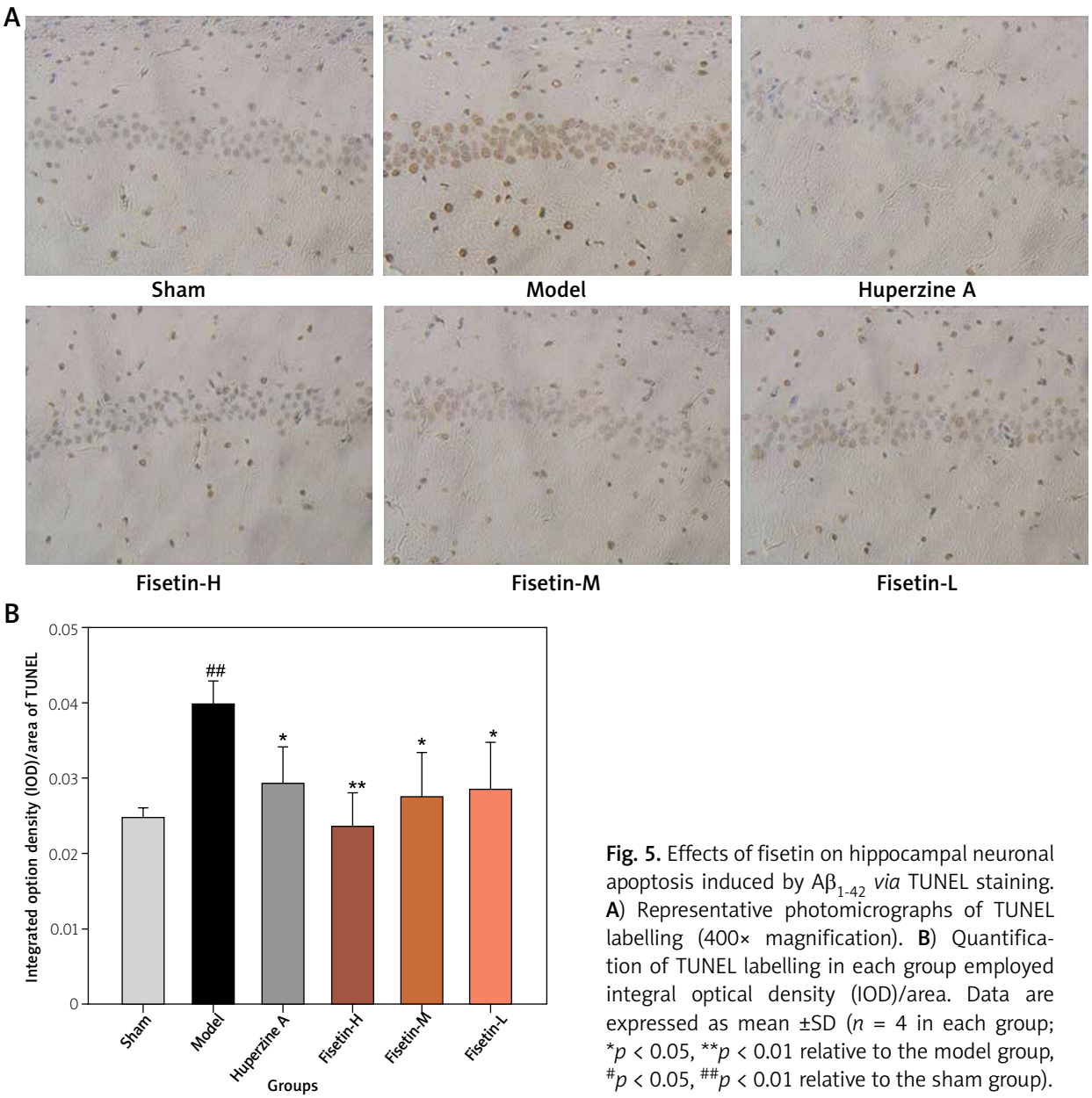

Fig. 5. Effects of fisetin on hippocampal neuronal apoptosis induced by $A\beta_{1\text{-}42}$ *via* TUNEL staining. **A**) Representative photomicrographs of TUNEL labelling (400× magnification). **B**) Quantification of TUNEL labelling in each group employed integral optical density (IOD)/area. Data are expressed as mean ±SD (n = 4 in each group; $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ relative to the sham group).

cholinergic dysfunction, inflammatory responses, neuronal apoptosis, and memory impairment. Given this host of Aβ-pathophysiologies, we sought to determine whether the known neuroprotective and anti-inflammatory properties of fisetin would have any ameliorative effects. Thus, we utilized an Aβ-induced rat model of AD to investigate the protective effects of fisetin on the pathogenesis and progression of AD.

To investigate the neuroprotective effects of fisetin on the hippocampus-dependent memory induced by $A\beta_{1\text{-}42}$, the Morris water maze and passive avoidance tests were used. First, we found that fisetin could significantly ameliorate $A\beta_{1\text{-}42}$-induced spatial learning and memory impairment in rats. This was particularly true at higher doses (50 and 100 mg/kg). These advantageous effects of fisetin on the learning and memory ability of AD rats may be due to its ability to decrease Aβ-induced damage to the brain.

We next sought to better understand the mechanism behind the basis of Aβ-induced neurotoxicity. Although the mechanism underlying Aβ-induced neurotoxicity is complex, it is known that increased oxidative stress may augment its neurotoxic effects and enhance neuron apoptosis [38]. Here, we investigated whether the activities of GSH-Px and T-SOD, the levels of MDA as well as 8-OHdG in the hippocampus would

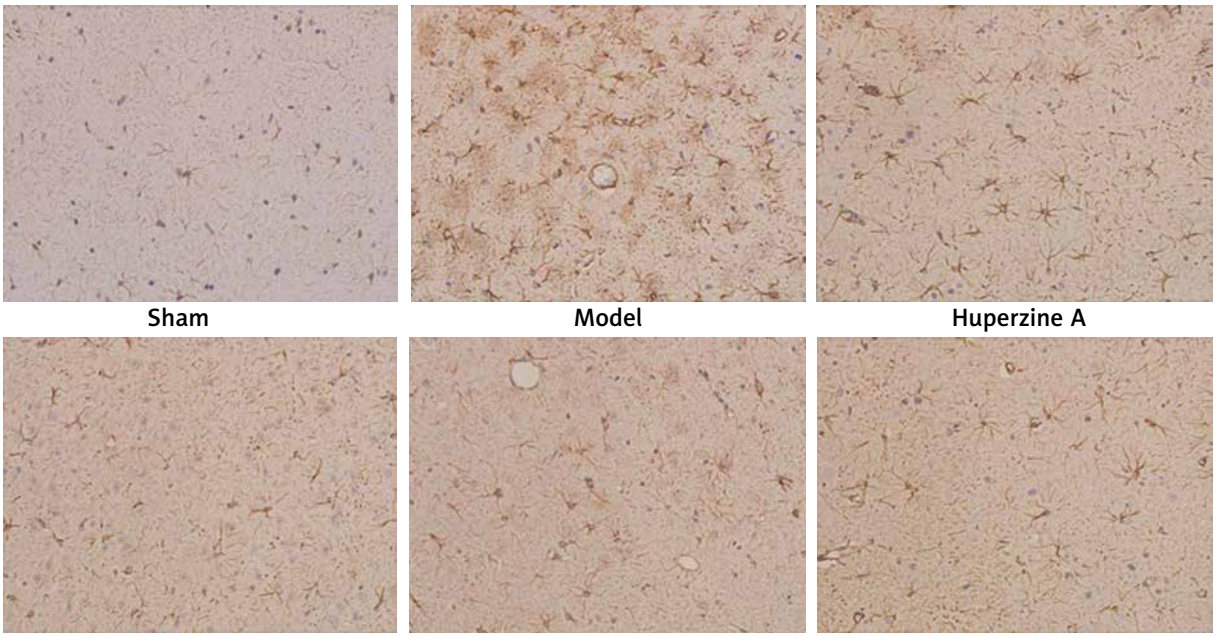

Fig. 6. Effects of fisetin treatment on the level of GFAP after Aβ1-42 treatment in the CA1 region of the hippocampus. Immunohistochemical assay of GFAP in the CA1 region of the hippocampus for each group. Representative photomicrographs of GFAP labelling in the hippocampus (400× magnification; *n* = 4 in each group).

be altered as a function of fisetin treatment. We found that fisetin doses of 50 and 100 mg/kg enhanced both GSH-Px and T-SOD activities in addition to decreasing the levels of MDA and 8-OHdG. These alterations could result in more efficacious scavenging of free radicals, ultimately leading to a reduction of Aβ-induced oxidative damage. This result was in agreement with our immunohistochemical staining data (H&E, TUNEL), which showed that administration of fisetin (1) significantly ameliorated exterior morphological characteristics of hippocampal neurons and (2) reduced the number of $A\beta_{1-42}$-induced apoptotic neurons. Thus, antioxidative mechanisms are likely important to the ability of fisetin to reduce Aβ-induced hippocampal memory deficits and neurodegeneration.

An important phenomenon in AD is the dysfunction of the cholinergic system in areas like the cortex and hippocampus [9]. The loss of cholinergic neurons and changes to ChAT and AChE activities are closely associated with the reduction of acetylcholine synthesis, which are associated with memory problems in AD [29]. In addition, it is widely accepted that astrocyte- and microglia-mediated inflammation contributes to the progression of AD. In the AD mice, astrocytes and microglia were activated and clustered around dense-core amyloid plaques, which can promote synaptic loss, cognitive dysfunction, and, eventually, neuronal death [14,17]. To this end, GFAP is an intermediate filament protein that is primarily expressed in astrocytes. Previous research has indicated that Aβ were surrounded by reactive astrocytes that have increased GFAP expression in $A\beta_{42}$-induced AD mice models [13]. Moreover, apoptosis-related caspases have been proposed to be significant contributors to the progressive neuronal death seen in AD. More specifically, caspase-3 is considered a principal effector caspase in apoptotic cascades and has been shown that Aβ can induce neuronal apoptosis *via* caspase-3 activation [11,27]. Abnormal intracellular accumulation and extracellular Aβ deposition in AD leads to the amplification of oxidative stress, which is itself a trigger for a series of inflammatory reactions and apoptosis that are implicated in its pathogenesis [5,7]. Post-mortem analysis of AD brains has revealed the presence of many activated microglia and astrocytes [26]. Notably, GFAP primarily expressed in astrocytes, correlated strongly with Aβ markers and with all assessments of cognition, which suggests that GFAP could emerge as the most robust indicator of disease progression, tau/Aβ pathology, and cognitive impairment [3]. Moreover, apoptosis-related caspases have been proposed to significantly contribute to neuronal death in AD [11]. Specifically, caspase-3 has been shown to be a main effector caspase in the apoptotic cascade(s) leading to neuronal apoptosis [11]. Here, we examined GFAP and caspase-3 expression in an AD rat model. Our research demonstrated that GFAP and caspase-3 expression were both elevated in the AD rat

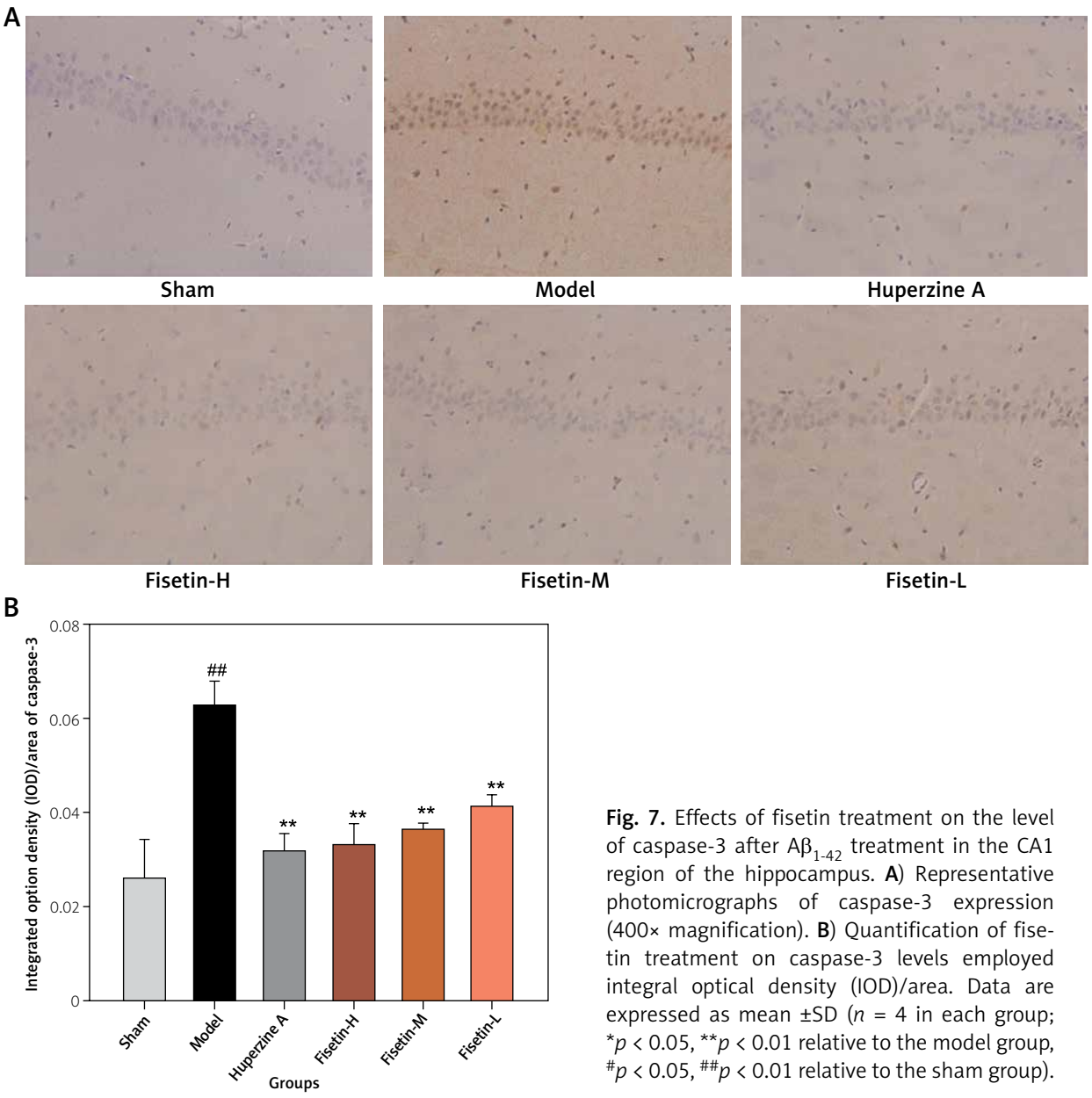

Fig. 7. Effects of fisetin treatment on the level of caspase-3 after $A\beta_{1\text{-}42}$ treatment in the CA1 region of the hippocampus. **A**) Representative photomicrographs of caspase-3 expression (400× magnification). **B**) Quantification of fisetin treatment on caspase-3 levels employed integral optical density (IOD)/area. Data are expressed as mean ±SD (n = 4 in each group; $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ relative to the sham group).

model, but that fisetin treatment resulted in significant reductions. This was particularly apparent at doses of 25, 50, and 100 mg/kg.

Finally, we also examined changes to the cholinergic system in the AD rat model, and selected both ChAT and AChE activities in the hippocampus. Our results showed that the activity of ChAT was significantly decreased while that of AChE was significantly increased in our $A\beta_{1\text{-}42}$-induced model group. Importantly, the activities of ChAT in the Fisetin-H, Fisetin-M, and Fisetin-L treatment groups were significantly enhanced and the activities of AChE in both Fisetin-H and Fisetin-M groups were all significant reduced. We found that the lowest dose of fisetin (Fisetin-L) has no significant effect on AChE levels.

Fisetin is a flavonoid that reduces free radical release, enhances mitochondrial function, and alleviates renal ischemia-reperfusion injury through antioxidant effects [21]. It has been reported that fisetin can block the activation of the NLRP3 inflammasome, inhibit the secretion of IL-1β into the central nervous system, and suppress neuroinflammation in rats with sepsis-associated encephalopathy [6]. Fisetin can also have an anticancer effect by enhancing apoptosis and inhibiting

cancer cell division, proliferation and invasion [33]. This study elucidated that fisetin attenuates $A\beta_{1-42}$-induced learning and memory impairments by inhibiting oxidative stress, adjusting cholinergic dysfunction, and its anti-inflammatory and apoptotic properties.

Conclusions

Collectively, this study sought to assess the effects of fisetin on ameliorating $A\beta_{1-42}$-induced learning and memory impairments in a rat model of AD. We also investigated a possible mechanism behind the effects of fisetin on inhibiting oxidative stress, cholinergic dysfunction, and the expression of GFAP and caspase-3. We found that there were some differences in the effects of fisetin and its mechanism within the range of our three tested doses. Fisetin-L was able to effectively increase the activities of GSH-Px and ChAT, which would give it both antioxidant properties and result in the generation of acetylcholine (ACH). Fisetin-H was able to markedly reduce the level of MDA and the activities of AChE, allowing it to scavenge for oxidation product(s) and reduce the hydrolysis of acetylcholine. Finally, Fisetin-M was able to do both. Furthermore, the three fisetin doses were able to reverse the inflammatory response and apoptosis produced by $A\beta_{1-42}$-treatment. This was evidenced in a significant decrease in the expression of both GFAP and caspase-3 levels. Taken together, these findings suggest that fisetin is a potential agent for use in the treatment of AD and that it operates by inhibiting oxidative stress, adjusting cholinergic dysfunction, and its anti-inflammatory and apoptotic properties.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 11975048]; Beijing Union University Open Project [grant number SWHX202103] and Beijing Union University scientific research project funding [grant number XP202007].

Acknowledgements

We thank all participants, Beijing Union University.

Disclosure

The authors report no conflict of interest.

References

1. Alzheimer's Association. 2022 Alzheimer's disease facts and figures. Alzheimers Dement 2022; 18: 700-789.
2. Berron D, Vogel JW, Insel PS, Pereira JB, Xie L, Wisse LEM, Yushkevich PA, Palmqvist S, Mattsson-Carlgren N, Stomrud E, Smith R, Strandberg O, Hansson O. Early stages of tau pathology and its associations with functional connectivity, atrophy and memory. Brain 2021; 144: 2771-2783.
3. Buchanan H, Mackay M, Palmer K, Tothová K, Katsur M, Platt B, Koss DJ. Synaptic loss, ER stress and neuro-inflammation emerge late in the lateral temporal cortex and associate with progressive tau pathology in Alzheimer's disease. Mol Neurobiol 2020; 57: 3258-3272.
4. Chen F, Yang D, Cheng XY, Yang H, Yang XH, Liu HT, Wang R, Zheng P, Yao Y, Li J. Astragaloside IV ameliorates cognitive impairment and neuroinflammation in an oligomeric Aβ induced Alzheimer's disease mouse model via inhibition of microglial activation and NADPH oxidase expression. Biol Pharm Bull 2021; 44: 1688-1696.
5. Chiu YJ, Lin CH, Lee MC, Hsieh-Li HM, Chen CM, Wu YR, Chang KH, Lee-Chen GJ. Formulated Chinese medicine Shaoyao Gancao Tang reduces NLRP1 and NLRP3 in Alzheimer's disease cell and mouse models for neuroprotection and cognitive improvement. Aging 2021; 13: 15620-15637.
6. Ding H, Li Y, Chen S, Wen Y, Zhang S, Luo E, Li X, Zhong W, Zeng H. Fisetin ameliorates cognitive impairment by activating mitophagy and suppressing neuroinflammation in rats with sepsis-associated encephalopathy. CNS Neurosci Ther 2022; 28: 247-258.
7. Ding Y, Zhang H, Liu Z, Li Q, Guo Y, Chen Y, Chang Y, Cui H. Carnitine palmitoyltransferase 1 (CPT1) alleviates oxidative stress and apoptosis of hippocampal neuron in response to beta-amyloid peptide fragment Aβ25-35. Bioengineered 2021; 12: 5440-5449.
8. Futamura A, Hieda S, Mori Y, Kasuga K, Sugimoto A, Kasai H, Kuroda T, Yano S, Tsuji M, Ikeuchi T, Irie K, Ono K. Toxic amyloid-β42 conformer may accelerate the onset of Alzheimer's disease in the preclinical stage. J Alzheimers Dis 2021; 80: 639-646.
9. Geula C, Dunlop SR, Ayala I, Kawles AS, Flanagan ME, Gefen T, Mesulam MM. Basal forebrain cholinergic system in the dementias: Vulnerability, resilience, and resistance. J Neurochem 2021; 158: 1394-1411.
10. Goh XX, Tang PY, Tee SF. 8-Hydroxy-2'-deoxyguanosine and reactive oxygen species as biomarkers of oxidative stress in mental illnesses: a meta-analysis. Psychiatry Investig 2021; 18: 603-618.
11. Günther A, Luczak V, Abel T, Baumann A. Caspase-3 and GFAP as early markers for apoptosis and astrogliosis in shRNA-induced hippocampal cytotoxicity. J Exp Biol 2017; 220: 1400-1404.
12. Huang Z, Ji H, Shi J, Zhu X, Zhi Z. Engeletin attenuates Aβ1-42-induced oxidative stress and neuroinflammation by Keap1/Nrf2 pathway. Inflammation 2020; 43: 1759-1771.
13. Ikram M, Muhammad T, Rehman SU, Khan A, Jo MG, Ali T, Kim MO. Hesperetin confers neuroprotection by regulating Nrf2/TLR4/NF-κB signaling in an Aβmouse model. Mol Neurobiol 2019; 56: 6293-6309.
14. Katsouri L, Birch AM, Renziehausen AWJ, Zach C, Aman Y, Steeds H, Bonsu A, Palmer EOC, Mirzaei N, Ries M, Sastre M. Ablation of reactive astrocytes exacerbates disease pathology in a model of Alzheimer's disease. Glia 2020; 68: 1017-1030.
15. Khan N, Syed DN, Ahmad N, Mukhtar H. Fisetin: a dietary antioxidant for health promotion. Antioxid Redox Signal 2013; 19: 151-162.
16. Li LX, Liu MY, Jiang X, Xia ZH, Wang YX, An D, Wang HG, Heng B, Liu YQ. Metformin inhibits Aβ25-35-induced apoptotic cell death in SH-SY5Y cells. Basic Clin Pharmacol Toxicol 2019; 125: 439-449.

17. Liu L, Liu Y, Li N, Huang R, Zheng X, Huang L, Hou S, Yuan Q. Multiple inflammatory profiles of microglia and altered neuroimages in APP/PS1 transgenic AD mice. Brain Res Bull 2020; 156: 86-104.
18. Majdi A, Sadigh-Eteghad S, Rahigh Aghsan S, Farajdokht F, Vatandoust SM, Namvaran A, Mahmoudi J. Amyloid-β, tau, and the cholinergic system in Alzheimer's disease: seeking direction in a tangle of clues. Rev Neurosci 2020; 31: 391-413.
19. Omari Shekaftik S, Nasirzadeh N. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) as a biomarker of oxidative DNA damage induced by occupational exposure to nanomaterials: a systematic review. Nanotoxicology 2021; 15: 850-864.
20. Palombi O, Shin JW, Watson C, Paxinos G. Neuroanatomical affiliation visualization-interface system. Neuroinformatics 2006; 4: 299-317.
21. Prem PN, Kurian GA. Fisetin attenuates renal ischemia/reperfusion injury by improving mitochondrial quality, reducing apoptosis and oxidative stress. Naunyn Schmiedebergs Arch Pharmacol 2022; 395: 547-561.
22. Raskatov JA. What is the "relevant" amyloid β42 concentration? Chembiochem 2019; 20: 1725-1726.
23. Reiss AB, Arain HA, Stecker MM, Siegart NM, Kasselman LJ. Amyloid toxicity in Alzheimer's disease. Rev Neurosci 2018; 29: 613-627.
24. Ribeiro S, Sharma R, Gupta S, Cakar Z, De Geyter C, Agarwal A. Inter- and intra-laboratory standardization of TUNEL assay for assessment of sperm DNA fragmentation. Andrology 2017; 5: 477-485.
25. Sarlak Z, Moazzami M, Attarzadeh Hosseini M, Gharakhanlou R. The effects of aerobic training before and after the induction of Alzheimer's disease on ABCA1 and APOE mRNA expression and the level of soluble Aβ-42 in the hippocampus of male Wistar rats. Iran J Basic Med Sci 2019; 22: 399-406.
26. Taipa R, Ferreira V, Brochado P, Robinson A, Reis I, Marques F, Mann DM, Melo-Pires M, Sousa N. Inflammatory pathology markers (activated microglia and reactive astrocytes) in early and late onset Alzheimer disease: a post mortem study. Neuropathol Appl Neurobiol 2018; 44: 298-313.
27. Takada E, Okubo K, Yano Y, Iida K, Someda M, Hirasawa A, Yonehara S, Matsuzaki K. Molecular mechanism of apoptosis by amyloid β-protein fibrils formed on neuronal cells. ACS Chem Neurosci 2020; 11: 796-805.
28. Tuerxun M, Muhda A, Yin L. The molecular mechanisms of signal pathway activating effect of E2F-1/NF-βB/GSK-3βon cognitive dysfunction of Alzheimer rats. Bioengineered 2021; 12: 10000-10008.
29. Varshney V, Garabadu D. Naringin exhibits mas receptor-mediated neuroprotection against amyloid beta-induced cognitive deficits and mitochondrial toxicity in rat brain. Neurotox Res 2021; 39: 1023-1043.
30. Vorhees CV, Williams MT. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 2006; 1: 848-858.
31. Wei L, Lv S, Huang Q, Wei J, Zhang S, Huang R, Lu Z, Lin X. Pratensein attenuates Aβinduced cognitive deficits in rats: enhancement of synaptic plasticity and cholinergic function. Fitoterapia 2015; 101: 208-217.
32. Wu MF, Yin JH, Hwang CS, Tang CM, Yang DI. NAD attenuates oxidative DNA damages induced by amyloid beta-peptide in primary rat cortical neurons. Free Radic Res 2014; 48: 794-805.
33. Xiao Y, Liu Y, Gao Z, Li X, Weng M, Shi C, Wang C, Sun L. Fisetin inhibits the proliferation, migration and invasion of pancreatic cancer by targeting PI3K/AKT/mTOR signaling. Aging 2021; 13: 24753-24767.
34. Yasuno F, Nakamura A, Kato T, Iwata K, Sakurai T, Arahata Y, Washimi Y, Hattori H, Ito K. An evaluation of the amyloid cascade model using in vivo positron emission tomographic imaging. Psychogeriatrics 2021; 21: 14-23.
35. Zhan JQ, Chen CN, Wu SX, Wu HJ, Zou K, Xiong JW, Wei B, Yang YJ. Flavonoid fisetin reverses impaired hippocampal synaptic plasticity and cognitive function by regulating the function of AMPARs in a male rat model of schizophrenia. J Neurochem 2021; 158: 413-428.
36. Zhang HF, Zhang HB, Wu XP, Guo YL, Cheng WD, Qian F. Fisetin alleviates sepsis-induced multiple organ dysfunction in mice via inhibiting p38 MAPK/MK2 signaling. Acta Pharmacol Sin 2020; 41: 1348-1356.
37. Zhang P, Cui J. Neuroprotective effect of fisetin against the cerebral ischemia-reperfusion damage via suppression of oxidative stress and inflammatory parameters. Inflammation 2021; 44: 1490-1506.
38. Zheng L, Kågedal K, Dehvari N, Benedikz E, Cowburn R, Marcusson J, Terman A. Oxidative stress induces macroautophagy of amyloid beta-protein and ensuing apoptosis. Free Radic Biol Med 2009; 46: 422-429.
39. Zufferey V, Gunten AV, Kherif F. Interactions between personality, depression, anxiety and cognition to understand early stage of Alzheimer's disease. Curr Top Med Chem 2020; 20: 782-791.