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Neuroprotective Effects of an Aqueous Extract of Futokadsura Stem in an Aβ-Induced Alzheimer's Disease-Like Rat Model

Wen Xia[1], Da-Wei Li[2], Lan Xiang[3], Jun-Jing Chang[2], Zuo-Li Xia[2], and En-Ji Han[1]

[1]*Department of Neurology, Qilu Hospital of Shandong University*
[3]*Institute of Pharmacognosy, College of Pharmacy, Shandong University, Jinan 250012*
and
[2]*Institute of Life Science, Taishan Medical College, Taian 271000*
Shandong, People's Republic of China

Abstract

Futokadsura stem has been traditionally used to dispel wind-damp obstruction syndrome manifested as painful and stiff joints, tendon and muscle spasms, lower back pain, painful knees and pain from external injury. An aqueous extract of Futokadsura stem has previously been found to have neuro-protective effects *in vitro*. In this study, we aimed to investigate if the Futokadsura stem extract could protect the neuron in the brain of an Aβ-induced Alzheimer's Disease (AD)-like rat model by improving the learning and memory ability of the rats. Learning and memory ability of the rats were measured by the Morris water maze test. Neuronal morphology in the hippocampus was examined by HE staining. Expression levels of Aβ, TNF-α, IL-6 and synaptophysin (SYP) were measured by immunofluorescence. Nitric oxide (NO) and nitric oxide synthase (NOS) levels were measured by NO and NOS detecting kit. We found that aqueous extract of Futokadsura stem alleviated Aβ(25-35)-induced impairment of spatial learning and memory in the AD rats. Furthermore, the extract protected the neurons by decreasing the expression of Aβ, TNF-α and IL-6 and the content of NO and NOS in the brain, and increasing the expression of SYP in the hippocampus. Our data indicated that aqueous extract of Futokadsura stem improved the learning and memory ability of AD rats. The neuro-protective effect was accomplished probably by depressing inflammation and oxidative stress in the brain.

Key Words: Alzheimer disease, Futokadsura stem, inflammation, oxidative stress, synaptophysin

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with global mental dysfunction and impairment of cognitive function (17). The pathologic features of AD are complex, including cerebral accumulation of extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFT) as well as loss of synaptic connections within selective brain regions (2). SP is extracellular accumulations of amyloid beta (Aβ) peptides that are derived from abnormal proteolytic processing of the amyloid precursor protein (APP); NFT is formed by intraneuronal accumulations of insoluble and hyper-phosphorylated tau, a microtubule-binding protein (11). Although the exact etiologies of AD still remain elusive, considerable evidences gained over the past decade have supported that neuroinflammation and oxidative stress may contribute to the pathogenesis of AD. In particular, Aβ(25-35) seems to be responsible for neuroinflammation and oxidative events leading to brain damage, such as activation of microglia and astrocytes (27), oxidative stress-mediated changes in hippocampal long-term potentiation (26), protein nitration, induction of

Corresponding author: Professor En-Ji Han, Department of Neurology, Qilu Hospital of Shandong University, Jinan 250012, Shandong, People's Republic of China. Tel: +0086-0531-82169341, E-mail: enjihan6@gmail.com
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inducible nitric oxide synthase (1, 25) and protein oxidation in fibroblasts derived from AD patients (4).

Aβ(25-35) can be produced in AD patients by enzymatic cleavage of the naturally occurring Aβ(1-40) (8). Abundant reports support that Aβ(25-35) is an active partial fragment of Aβ. This fragment also forms a β-sheet structure (18) and induces neuronal cell death (18, 30), neuritic atrophy (21) and synaptic loss (7, 23, 24). Intrahippocampal or intracerebroventricular (i.c.v.) injections of Aβ(25-35) induced histological and biochemical changes, learning deficits (1, 15, 16) and dysfunction of the cholinergic system (25). Thus, Aβ(25-35) injected animals are useful models for understanding the pathogenesis and progression of AD, and for evaluating new therapeutic agents for AD (15).

Futokadsura stem is the caulis of *piper futokadsura* Sieb, et Zucc. The traditional function of the plant is to dispel wind-damp obstruction syndrome manifested as painful and stiff joints, tendon and muscle spasms, lower back pain, painful knees and pain from external injury. Kadsurenone, a monomer component isolated from Futokadsura stem, was found to be a specific platelet-activating factor receptor antagonist (22). Futokadsura stem is now being used for therapy of ischemic stroke, arthralgia and asthma. In previous studies, we have found that aqueous extracts of Futokadsura stem could selectively inhibit the expression of APP gene at the mRNA and protein levels in SK-N-SH neuroblastoma cells (9, 10). Since Aβ(25-35), which is derived from APP, is responsible for neuroinflammation and oxidative events leading to brain damage and neurodegeneration, we hypothesize that aqueous extracts of Futokadsura stem can ameliorate the neuroinflammation and oxidative stress in the brain of Aβ(25-35) i.c.v. injected AD model rats to prevent the synapse degeneration, and consequently, improve the learning and memory ability of the rats.

Materials and Methods

Animals

Male Wistar rats weighing about 250-300 g were obtained from the Experimental Animal Center of Shandong University, Jinan, PRC, and housed in cages at room temperature (25°C) with alternating 12:12h light-dark cycles. Standard rat chow pellets and water were supplied *ad libitum.* Behavioral experiments were carried out in a sound-proof and airregulated experimental room, to which rats were habituated for at least 1 h. Rats were randomly divided into 6 groups (n = 10 each group): normal control group (NC), model control group (MC), sham group (Sham), positive control group (Ibuprofen), high dose futokadsura group (HFT-1) and low dose futokadsura group (HFT-2). After acclimatization to the laboratory conditions for 1 week, rats from the NC group received no treatment, rats from the MC, Ibuprofen, HFT-1 and HFT-2 groups received lateral ventrical injection of Aβ(25-35) at a dose of 10 μg, and rats from the Sham group were injected with the same volume of normal saline. One week after injection, rats in the Ibuprofen group were orally administered with Ibuprofen at 200 mg/kg per day for 2 weeks, rats in the HFT-1 and HFT-2 groups were orally administered with aqueous extract of futokadsura stem at 1400 mg/kg per day and 460 mg/kg per day respectively), rats in the NC group received the same volume of distilled water. After 2 weeks of drug administration, rats in all the groups received Morris water maze (MWM) test. This study was approved by the Animal Ethics Committee of Qilu Hospital, Shandong University.

Preparation of Aqueous Extract of Futokadsura Stem

100 g dried crude drug of Futokadsura stem was pulverized and extracted with 500 ml distilled water. After 1 h immersion, it was boiled for 30 min and then filtered. After adding 500 ml distilled water, it was boiled for another 20 min, and the filtrates were pooled. The filtrates were concentrated by a rotary evaporator, and dried in a lyophilizer. The lyophilized powder was stored at 4°C in the dark.

Intracerebroventricular(I.c.v.) Injection

Aβ(25-35) was dissolved in physiological saline at a concentration of 1 μg/μl and was incubated in distilled water at 37°C for 5 days to allow fibril formation. Under general anesthesia with 10% chloral hydrate (300 mg/kg), 10 μg fibrillar Aβ(25-35) (10 μl) or vehicle (saline, 10 μl) was injected into the CA1 area of the dorsal right hippocampus at the following coordinates: anterior posterior, –3.6 mm; lateral, 2.4 mm; horizontal, 2.8 mm from the bregma, according to the atlas by Paxinos and Watson. Aβ(25-35) (10 μl) or vehicle (saline,10 μl) was delivered gradually within 3 sec. Rats exhibited normal behavior within 1 min after injection. In preliminary experiments, the injection site was confirmed by injecting Indian ink. Neither insertion of the needle nor injection of saline had a significant influence on survival, behavioral responses or cognitive functions.

Morris Water Maze Test

Seven days after the Aβ(25-35) injection, rats in each group were administered drugs. The Morris water maze test was carried out on the 14th day after drug administration using the Morris Water Maze Tracking System (BI-2000, TME; Chengdu, PRC) by the method

of Morris with minor modifications. Briefly, a white circular tank (120 cm in diameter and 50 cm high) with a featureless inner surface was used. The pool was filled to a depth of 30 cm with opaque water (23 ± 2°C). A white platform (9 cm in diameter) was submerged 1 cm below the water surface and placed at the midpoint of a fixed quadrant. Memory-acquisition trials (training) were performed six times daily for 5 days and escape latencies (time of finding submerged platform) were observed. On each trial, the rats were allowed to swim freely until they found the platform and left for an additional 10 s on the platform after they found the platform. However, the rats failing to find the platform within 120 sec were placed on the platform manually for 10s and escape latencies were considered as 120 sec. Memory retention tests were made by removing the platform and allowing each rat to swim freely for 120 sec inside the pool in the sixth day, and the number of crossings over a point where the platform had been recorded.

Immunofluorescence

After the Morris water maze test, the rats were perfused through the ascending aorta with 100 ml cold normal saline followed by 100 ml 4% paraformaldehyde (PFA) in PBS. Brains were removed and postfixed in the same fixative for 3 days followed by 30% sucrose for 1 week. Sections were cut at a 12 μm thickness in a freezing microtome and stored at -20°C. For immunostaining, tissue sections were fixed with 4% PFA for 10 min. After several washes in PBS, the sections were incubated with blocking buffer containing 0.3% Triton X-100 and 4% bovine serum albumin for 1 h at room temperature, and were then stained with the desired primary antibody reconstituted in PBS, 2% goat serum at 4°C for 14-16 h. Anti-TNF-α (Boster Bio-engineering Company Limited, Wuhan, PRC), anti-IL-6 (Boster Bio-engineering) and anti-synaptophysin (Santa Crus Biotechnology, Dallas, Texas, USA) antibodies were dilated at 1:300. After three rinses in PBS, sections were incubated with goat anti-rabbit IgG FITC conjugate (Boster Bio-engineering) at 1:100 dilution for 1 h at room temperature. After several washes in PBS, sections were mounted with Crystal Mount and analyzed using a laser-scanning confocal microscope (Radiance 2100, Bio-Rad, Hercules, CA, USA). Fluorescence intensities of immuno-positive areas (after subtracting the background intensity) in the hippocampus were quantified using an image analysis system (Lasersharp 2000, Bio-Rad). The relative position of Aβ, TNF-α, IL-6 or synaptophysin was manifested by nucleus red fluorescence stained by propidium iodide (PI).

HE Staining

For pathological studies, after being pre-fixed in 10% methanal for 24 h, the right cerebral hemisphere was postfixed in 70% ethanol for at least 12 h. Having been dehydrated, the brain was embedded in paraffin blocks. Coronal blocks embedded in paraffin were serially cut for 5 μm thick coronal sections through the visual cortex, hippocampus and stained with HE. Each brain section was observed with a light microscope. Cells with a round or oval-shaped nuclei were regarded as undamaged. The number of undamaged cells was counted under a light microscope at a magnification of 400X using a computer-associated image analyzer (Olympus, Japan).

Determination of NO and NOS Content

Protein levels of NO and NOS in hippocampal tissues were measured by NO and NOS detecting kit (Jiancheng Bioengineering Institute, Nanking, PRC) according to the manufacturer's instructions.

Statistical Analyses

The data were expressed as mean ± SE. Statistical analysis of group differences was performed using Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Effects of Aqueous Extract of Futokadsura Stem on Morris Water Maze Test

To observe the effects of the aqueous extract of Futokadsura stem on Aβ(25-35)-induced spatial learning and memory impairment in AD rats, Morris water maze test was performed. The escape latency of finding a platform in the water maze was used as a measure for evaluating spatial memory, and reduction in escape latency from day to day reflected learning with respect to long-term memory. Results showed that escape latencies were significantly longer in rats with Aβ(25-35) treatment than that of the sham and normal control rats ($P < 0.05$) (Fig. 1A). In contrast, treatment with the Futokadsura stem extract and the positive control drug Ibuprofen, significantly shortened escape latencies in Aβ(25-35)-treated rats ($P < 0.05$). A significant decrease in the number of crossing over a platform position was recorded in the Aβ(25-35)-treated rats, compared with the sham and normal control rats ($P < 0.05$), and recovered by treatment with the Futokadsura stem extract and Ibuprofen in the Aβ(25-35)-treated rats ($P < 0.05$) (Fig. 1B). These results indicate that i.c.v. injection of Aβ(25-35) had led to impairment in spatial learning and memory, and treatment with the Futokadsura stem

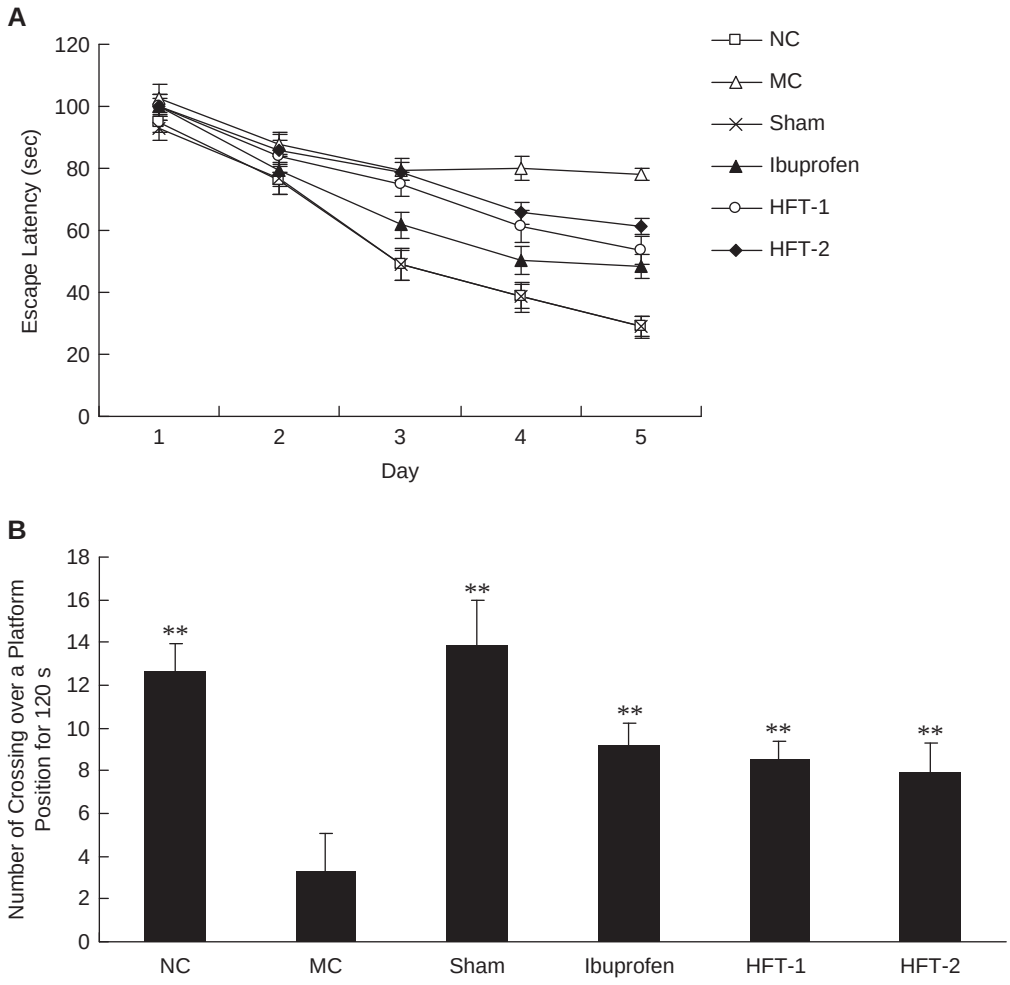

Fig. 1. Effects of Aβ(25-35) injection and Futokadsura stem extract treatment on (A) escape latency in hidden-platform acquisition and (B) on the number of crossing over a position where a platform had been in the water maze. Each value represents mean ± SEM (n = 10 rats per group). NC: normal control group; MC: model control group; Sham: sham group; Ibuprofen: positive control group; HFT-1: high dose Futokadsura stem group; HFT-2: low dose Futokadsura stem group. $^{**}P < 0.01$ *vs.* MC.

extract significantly ameliorated Aβ(25-35)-induced memory impairment (Fig. 1).

Effects of Aqueous Extract of Futokadsura Stem on the Neuronal Morphology in the Hippocampus

To investigate the effects of the aqueous extract of Futokadsura stem on Aβ(25-35)-treated neuronal morphology change in hippocampus, HE staining was performed. A significant decrease in the density of healthy neuron cells in the CA1 region of the hippocampus was observed in the Aβ(25-35)-treated rats, compared with the sham and normal control rats (Fig. 2A). Typical neuropathological changes, including neuron loss and nucleus shrinkage or disappearance, were found in the CA1 of the hippocampus in Aβ(25-35)-treated rats. However, the decrease in the amount of healthy cells in Aβ(25-35)-treated rats was reversed significantly after the treatment with the Futokadsura stem extract and positive control drug (Ibuprofen), suggesting that administration of the aqueous extract of Futokadsura stem could attenuate the Aβ(25-35)-induced reduction of healthy neurons with normal morphological appearances in the hippocampus.

Effects of Aqueous Extract of Futokadsura Stem on Aβ Expression in Hippocampal Neurons

A significant increase in the amount of Aβ expression in hippocampal neurons was observed in the model control group compared with the normal control and the sham group ($P < 0.01$) (Fig. 3). The increase in the amount of Aβ expression in the Aβ(25-35)-induced rats was reversed significantly after treatment with a high dose (1400 mg/kg), a low dose (460 mg/kg) of the Futokadsura stem extract and Ibuprofen (200 mg/kg).

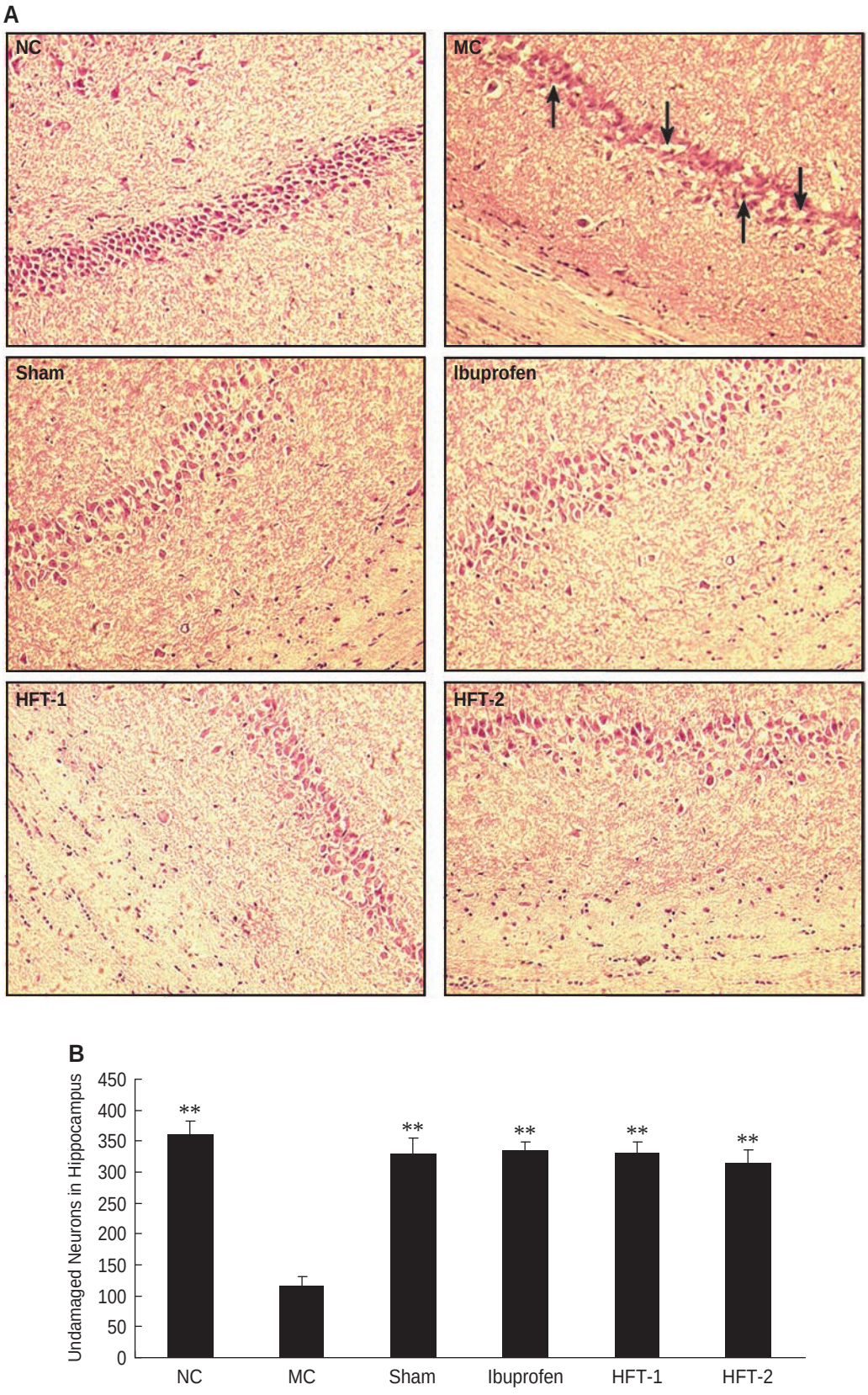

Fig. 2. Histochemical HE staining of the hippocampus in each group. (A) Representative micrographs of HE-positive cells in the CA1 region of the hippocampus (magnification, 200×). Arrows indicate damaged neurons. (B) Comparison of cell density of neurons in the hippocampus among different groups. Each value represents mean ± SEM (n = 10 rats per group). Group abbreviations are as in Fig. 1. $^{**}P < 0.01$ *vs*. MC.

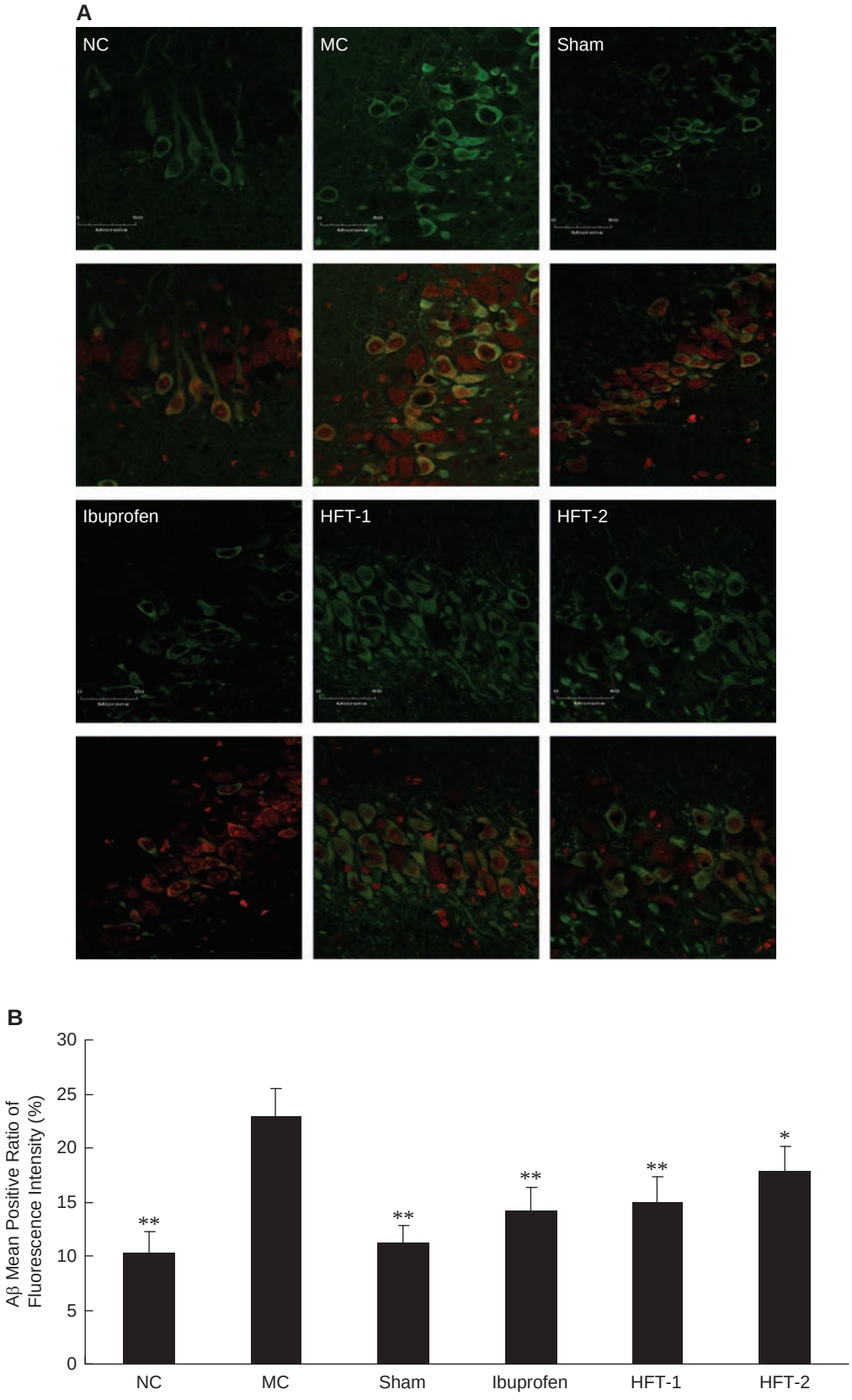

Fig. 3. Effects of Aβ(25-35) injection and Futokadsura stem extract treatment on Aβ expression in hippocampal neurons. (A) Representative micrographs of Aβ-positive cells (in green fluorescence) in the CA1 region of the hippocampus. The relative position of Aβ was manifested by nucleus red fluorescence stained by PI. (B) Mean positive ratio of fluorescence intensity of Aβ in different groups. Each value represents mean ± SEM (n = 10 rats per group). *$P < 0.05$ *vs*. MC, **$P < 0.01$ *vs*. MC.

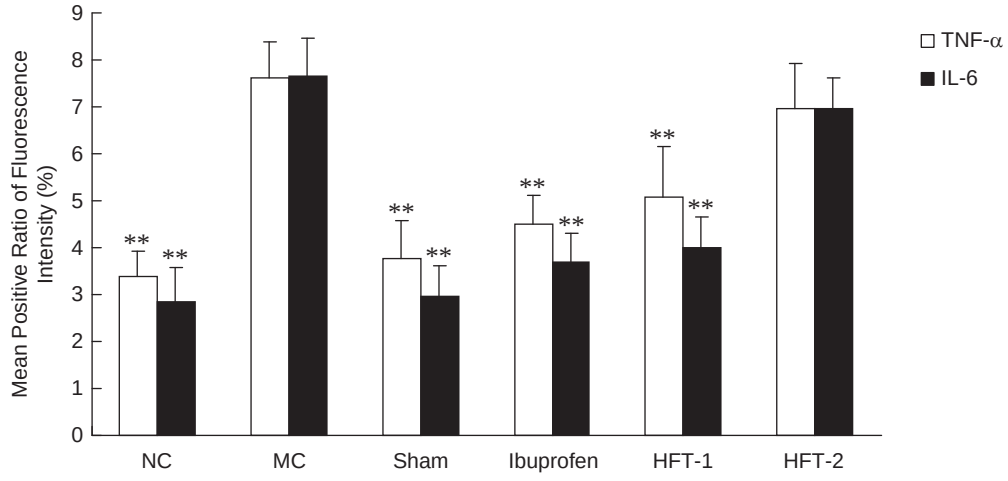

Fig. 4. Effects of Aβ(25-35) injection and Futokadsura stem treatment on TNF-α and IL-6 protein expression in the frontal glial cells. Mean positive ratio of fluorescence intensity of TNF-α and IL-6 in different groups. Each value represents mean ± SEM (n = 10 rats per group). $^{**}P < 0.01$ *vs*. MC.

Effects of Aqueous Extract of Futokadsura Stem on TNF-α and IL-6 Protein Expression in Frontal Glial Cells

A significant increase in the amount of TNF-α and IL-6 protein expression in frontal glial cells was observed in the model control group compared with the normal control group and sham group ($P < 0.01$) (Fig. 4). The TNF-α and IL-6 increases in the Aβ(25-35)-induced rats were significantly reduced after treatment with a high dose of the Futokadsura stem extract and Ibuprofen. However, the reduction was of no significance in the low dose Futokadsura stem group.

Effects of Aqueous Extract of Futokadsura Stem on the NO and NOS Levels

A significant increase in the NO and NOS levels was observed in the model control group compared with the normal control group and sham group ($P < 0.01$) (Fig. 5). The NO and NOS increases in the Aβ(25-35)-induced rats were significantly reduced after the treatment with high-dose Futokadsura stem extract and Ibuprofen). However, the reduction was not significant in the low dose group.

Effects of Aqueous Extract of Futokadsura Stem on Synaptophysin (SYP) Expression in Hippocampal Neurons

A significant decrease in the level of SYP expression in hippocampal neurons was observed in the model control group compared with the normal control group and sham group ($P < 0.01$) (Fig. 6). The decrease in the amount of SYP expression in the Aβ(25-35)-induced rats was significantly reversed after treatment with a high dose, a low dose of Futokadsura stem extract and Ibuprofen.

Discussion

In the present study, we showed that an aqueous extract of Futokadsura stem prevented Aβ(25-35)-induced impairment of spatial learning and memory in Morris water maze tests. Furthermore, Futokadsura stem extract decreased the expression of Aβ, TNF-α and IL-6 and the content of NO and NOS, increased the expression of synaptophysin (SYP) in the hippocampus after the Aβ(25-35) treatment. To our knowledge, this is the first study to show that aqueous extract of Futokadsura stem protects against Aβ induced neurotoxicity by depressing inflammation in the brain.

Amyloid precursor protein (APP) can be abnormally cleaved by β and γ-secretase to produce Aβ. Amyloid cascade hypothesis was first proposed by Hardy and Higgins (12). According to the hypothesis, missense mutations of APP or presenilin (familial AD) or failure of Aβ clearance (sporadic AD) lead to increased Aβ in the brain. Aβ soluble toxic oligomers (6, 28) are considered as a trigger signal for neurotoxicity, including a cascade of inflammatory reactions and oxidative stress. The inflammatory reactions are characterized by the aggregation of activated microglia around Aβ deposit sites in the brain and the release of proinflammatory mediators including proinflammatory cytokines such as IL-6 and TNF-α, proinflammatory synthases such as inducible nitric oxide synthase (iNOS), cycloxygenase-2 (COX-2), and so forth. Aβ disposition, activated glia, and upregulated inflammatory mediators constitute a feedback loop

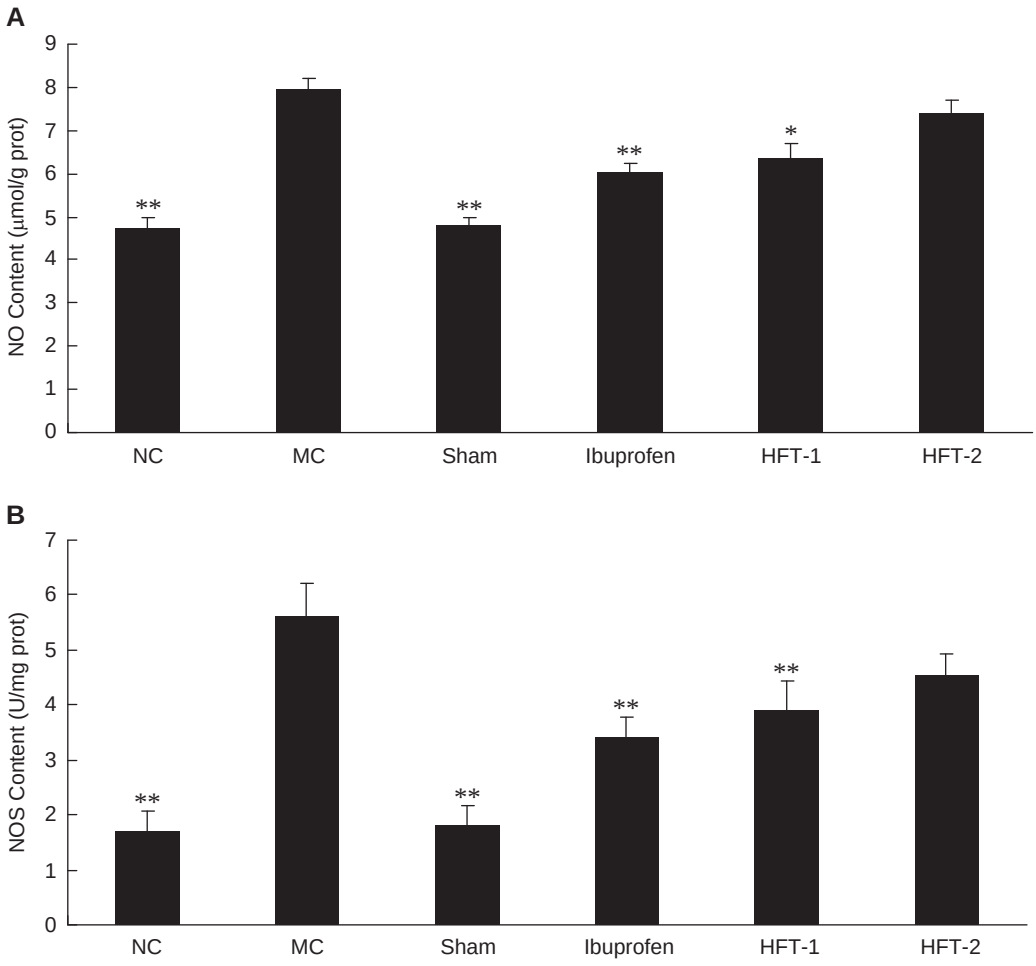

Fig. 5. Effects of Aβ(25-35) injection and Futokadsura stem treatment on NO and NOS in brain tissues of the rats. Each value represents mean ± SEM (n = 10 rats per group). *$P < 0.05$ *vs*. MC, **$P < 0.01$ *vs*. MC.

in which Aβ attracts and activates microglia, which have been shown previously to be a major source of inflammatory mediators, leading to the release of proinflammatory cytokines such as IL-6 and TNF-α, which in turn further increase the accumulation of Aβ and activation of microglia. This is a vicious cycle.

TNF-α is one of the main pro-inflammatory cytokines that plays a fundamental role in inflammatory responses (14). Elevated levels of TNF-α has been implicated in pathogenesis of Alzheimer disease (5). IL-6 promotes astrogliosis (20), activates microglia (13), and stimulates the production of acute phase proteins (3). Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS). It is also assumed to play a detrimental role in the pathological process of AD. Synaptic proteins are essential components to maintain normal synaptic function. Synaptophysin is the most abundant synaptic vesicle protein and is often used as a marker for quantifying the number of intact synapses.

In the previous studies, we have found that the aqueous extract of Futokadsura stem selectively inhibits the expression of APP and Aβ in mRNA and protein levels in SK-N-SH cells (9, 10, 31). In this study, the results further testified our hypothesis that Futokadsura stem extract could inhibit the expression of Aβ *in vivo*, and it could also ameliorate neuroinflammation, oxidative stress, synapse degeneration in the Aβ-induced AD-like rat model. The learning and memory ability in the rats were improved on treatment.

We have also previously showed that piperlonguminine/ dihydropiperlonguminine components isolated from Futokadsura stem inhibits the expression of APP and Aβ in SK-N-SH cells (19, 29). Do the APP and Aβ inhibition effects of the components occur *in vivo*? Do the components ameliorate the neuroinflammation, oxidative stress, synapse degeneration, or improve the learning and memory ability in the AD model ras? Are there other components in Futokadsura stem which have similar effects? These questions remain to be answered.

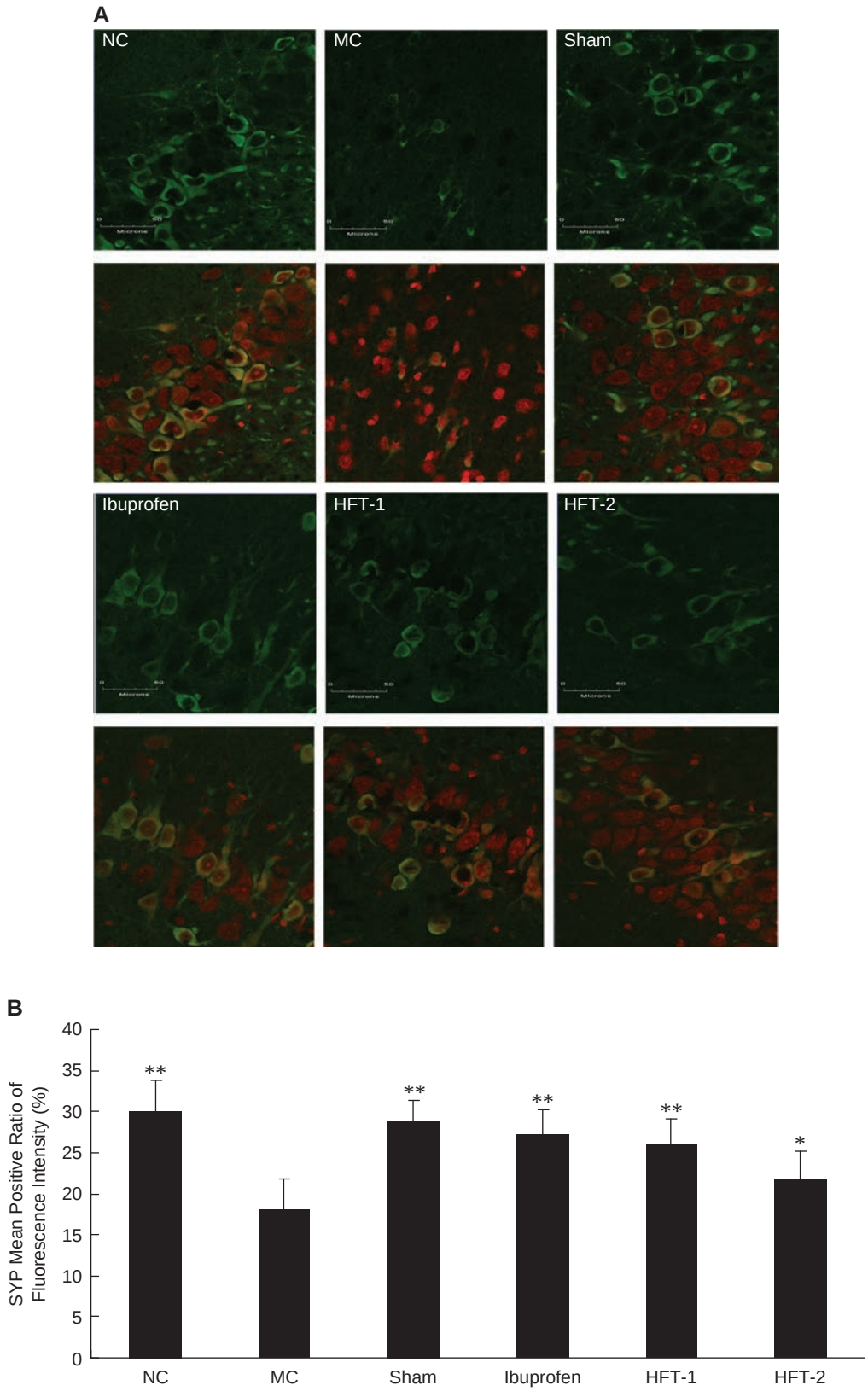

Fig. 6. Effects of Aβ(25-35) injection and Futokadsura stem treatment on synaptophysin (SYP) expression in hippocampus neurons. (A) Representative micrographs of SYP-positive cells (in green fluorescence) in the CA1 region of the hippocampus. The relative position of SYP was manifested by nucleus red fluorescence stained by PI. (B) Mean positive ratio of fluorescence intensity of SYP in different groups. Each value represents the mean ± SEM (n = 10 rats per group). *$P < 0.05$ *vs*. MC, **$P < 0.01$ *vs*. MC.

Acknowledgments

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