Protective Effects of Wu-Zi-Yan-Zong-Fang on Amyloid β-induced Damage *In Vivo* and *In Vitro*

Bin Wang, Xue Mei Wang,* Hong Fu, and Geng Xin Liu

Institute of Integration of Traditional Chinese and Western Medicine, Peking University First Hospital, No. 8, Xishiku Street, Xicheng District, 100034 Beijing, China

This study was designed to determine the effects of Wu-Zi-Yan-Zong-Fang on amyloid-β_{25-35}-induced cognitive deficits in rats and neurotoxicity in pheochromocytoma cells and the possible mechanism of action. *In vivo* studies showed that Wu-Zi-Yan-Zong-Fang significantly ameliorated the spatial memory and retention deficits, decreased acetylcholinesterase activity, and increased acetylcholine content caused by intracerebroventricular injection of amyloid-β_{25-35}. *In vitro* results showed that Wu-Zi-Yan-Zong-Fang increased cell viability and the activity of superoxide dismutase and catalase and decreased the release of lactate dehydrogenase and the level of malondialdehyde. Wu-Zi-Yan-Zong-Fang also significantly reduced the percentage of apoptotic cells and blocked the increase in the intracellular concentration of Ca^{2+}. These data suggest that Wu-Zi-Yan-Zong-Fang has potent protective effects for the treatment of Alzheimer's disease in future.

Key words——Alzheimer's disease; Wu-Zi-Yan-Zong-Fang; amyloid beta

INTRODUCTION

Alzheimer's disease (AD) is characterized by the deterioration of cognitive and mental functions, including learning and memory. The formation of extracellular deposits of amyloid beta peptide (Aβ) in the cortex and hippocampus is a prominent pathologic feature of AD.[1]

In animal experiments, intracerebroventricular (i.c.v.) injections of Aβ induced learning deficits and dysfunction of the cholinergic system.[2] The cognitive deficit associated with AD is suggested to be primarily related to defects of cholinergic neurotransmission in the brain. The inhibition of acetylcholinesterase (AChE), the metabolizing enzyme of acetylcholine (Ach), in the brain is important for increasing cholinergic neurotransmission. Oxidative stress, the imbalance between antioxidant capacity and reactive oxygen species (ROS) production, has been shown to precede pathologic lesions in AD.[3] ROS destroy cells by inducing apoptosis that may perturb the cell's natural antioxidant defense system.

Wu-Zi-Yan-Zong-Fang (WYF), a traditional Chinese medicine with five herbal ingredients, was first described in the 'XuanJieLu' in China in AD 1400. It has a long history in treating age-related symptoms, such as memory loss, talagia, and tinnitus. Our previous results demonstrated that WYF can markedly improve memory by increasing the volume of the hippocampus in patients with mild cognitive impairment.[4]

In the present study, we examined the effects of WYF on Aβ-induced memory impairment in rats. The Morris water maze (MWM) and probe trial paradigms were used to investigate short-term spatial memory and retention. We further examined the protective effects of WYF on the pheochromocytoma (PC12) cell lesion induced by Aβ and the possible mechanisms of action at the histologic level. These results may provide a pharmacologic basis for AD treatment with WYF.

MATERIALS AND METHODS

Chemicals Aβ_{25-35} was from Sigma-Aldrich Co. (Sunnyvale, CA, USA). Huperzine A (HupA) was from Taloph Pharmaceutical Enterprise (Henan, China). Newborn calf serum and RPMI-1640 medium were obtained from Gibco (NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). AchE, Ach, lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) assay kits were purchased from Nanjing Jiancheng Bioen-

*e-mail: wpku@hotmail.com

gineering Institute (Nanjing, China). The annexin V-FITC/PI apoptosis detection kit was obtained from Biosea BCL (Beijing, China). Fluo-3/AM and Pluronic F-127 were purchased from Biotium (Hayward, CA, USA). All other reagents were of analytical grade.

Preparation of WYF　Medicinal materials were purchased from Beijing Tongrentang Pharmacy (Beijing, China) and identified by Dr. P. F. Tu, Peking University School of Pharmaceutical Sciences, where a voucher specimen was deposited. WYF is comprised of five drugs: *Semen Cuscutae* (*tusizi*) (4000 g), *Fructus Lycii* (*gouqizi*) (4000 g), *Fructus Rubi* (*fupengzi*) (2000 g), *Fructus Schisandrae* (*wuweizi*) (500 g), and *Semen Plantaginis* (*cheqianzi*) (1000 g). The five medicinal materials were powdered and decocted for 1 h in 50 l of distilled water, and the residue was decocted again for 1 h in 50 l of distilled water. The filtrates were mixed and dried at 60℃. The yield of dried powder was 1800.76 g.

In Vivo Experiment

Animals　Adult male Sprague-Dawley (SD) rats (China Academy of Medical Sciences, Beijing, China) were housed 4 per cage on a 12-h light/12-h dark cycle with *ad libitum* access to rat chow and water. These animal experiments were carried out in accordance with recommendations from the Declaration of Helsinki and the internationally accepted principles for the experimental use of animals.

Microinjection Procedure　Referring to the atlas of Paxinos,[5] $A\beta_{25-35}$ was dissolved in sterile saline and incubated at 37℃ for 4 days. An injection cannula was inserted stereotaxically at a site 0.8 mm posterior and 1.5 mm lateral to the bregma and 4.0 mm below the surface of the cranium in the right lateral ventricle. Then 5 μl of sterile saline or 10 mM of $A\beta_{25-35}$ was injected. The injection needle was left in the guide cannula for an additional 60 s.

Drug Administration and Groups　After receiving an i.c.v. injection of either physiologic saline or $A\beta_{25-35}$ rats were orally administered the test compounds for 15 days prior to MWM testing and probe trial. The groups were: control ($n=10$); $A\beta_{25-35}$ 10 mM ($A\beta$, $n=10$); WYF 5 mg/kg+$A\beta_{25-35}$ 10 mM (WYF-5, $n=10$); WYF 50 mg/kg+$A\beta_{25-35}$ 10 mM (WYF-50, $n=10$); WYF 500 mg/kg+$A\beta_{25-35}$ 10 mM (WYF−500, $n=10$); and huperzine A 20 mg/kg+$A\beta_{25-35}$ 10 mM (HupA, $n=10$). The volume of injec-

tion was 1 ml/kg.

Apparatus　The water maze was a dark circular pool, 140 cm in diameter and 55 cm high, filled to a depth of 25 cm with water at a temperature of 24 ± 1 ℃. A goal platform (11 cm in diameter) was located 1.5 cm below the surface in the center of the northwest quadrant. The pool was placed in an experimental room furnished with several extra cues. The position of the animal was monitored with a camera.

MWM Test　This test was carried out following the method described by Morris *et al.*[6]

Probe Trial　This experiment was carried out as described by Rapp *et al.*[7] One day after the last MWM training trial, each rat was subjected to a probe trial (60 s) in which no platform was present. The percentage of time and length spent in the former platform quadrant was taken as a measure of spatial memory retention.

AchE and Ach Assays　AchE activity assays were carried out using an acetylthiocholine iodide substrate based on the colorimetric method as described by Ellman *et al.*,[8] with minor modifications.[9] After the MWM and probe test, the cerebral cortex and hippocampus were immediately dissected from the brain. Both parts of the brain were rapidly homogenized for the AchE assay.

Ach was determined according to the method of Vincent *et al.*[10] In brief, aliquots (0.8 ml) of the cerebral cortex and hippocampus homogenates were mixed with 1.4 ml of distilled water, 0.2 ml of physostigmine 1.5 mM, and 0.8 ml of trichloroacetic acid 1.84 M and blended thoroughly. After centrifugation, 1 ml of each supernatant was added to 1 ml of basic hydroxylamine, incubated for 15 min at 25℃, and then 0.5 ml of HCl 4 M and 0.5 ml of FeCl$_3$ 0.37 M were added. Absorbance was read at 540 nm and calibrated with a blank.

In Vitro Experiment

Cell Culture　PC12 cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, China) were grown in RPMI-1640 medium supplemented with 10% newborn calf serum at 37℃ in a humidified atmosphere of 5% CO$_2$ in air. All experiments were carried out 24 h after cells were seeded. HupA (20 μg/ml) served as a positive control.

Preparation of Fibrillar $A\beta_{25-35}$　$A\beta_{25-35}$ was dissolved in sterile saline at a concentration of 3 mM and stored at -20℃. To obtain the neurotoxic form of

Aβ_{25-35}, the peptide solution was placed in an incubator at 37°C for 4 days according to previous reports.[11]

MTT Assay   The MTT assay was carried out following the method described by Mosmann.[12] PC12 cells were plated in 96-well plates at a density of 1×10^4 cells/well. WYF (5, 50, and 500 μg/ml) was added 2 h before the addition of Aβ_{25-35}. After 24 h, MTT 0.5 mg/ml was added and incubated for 4 h at 37°C. Then the colored formazan was dissolved in DMSO. The absorbance at 570 nm was determined using a microplate spectrophotometer.

LDH, MDA, SOD, and CAT Assays   The LDH assay was carried out as described by Babson and Babson.[13] After treatment, the supernatant was collected for LDH measurement. The reaction mixture contained 2.90 μl of Tris buffer 0.2 M, supernatant 10 μl, 100 μl of sodium pyruvate 30 mM, and NADH 100 μl. The rate of change in absorbance was measured at 440 nm for 2 min at 30-s intervals. The oxidation of NADH in the reaction was proportional to LDH present the sample. LDH was determined from a standard curve obtained using commercially available LDH. LDH levels are expressed as units per liter.

PC12 cells were washed twice with ice-cold PBS (0.1 M, pH=7.4) and then centrifuged at 10000 g for 30 min at 4°C, and the resulting supernatant was stored at -70°C until MDA, SOD, and CAT analysis.

The MDA assay was performed as Yagi.[14] Briefly, 100 μl of homogenate was further centrifuged with 100 μl of 10% TCA at 5000 g for 20 min at 4°C. Subsequently, 0.2 μl of homogenate was mixed with 0.2 μl of 8.1% sodium dodecyl sulfate, 1.5 μl of 30% acetic acid (pH 3.5), and 1.5 μl of 0.8% thiobarbituric acid. The reaction mixture was heated for 60 min at 95 °C and then cooled. After cooling, 1.0 μl of distilled water and 5.0 μl of n-butanol: pyridine (15 : 1 v/v) were added and centrifuged at 5000 g for 20 min. The absorbance of the pink-colored organic layer was measured at 532 nm. 1,1,3,3-Tetraethoxypropane (Sigma Chemicals, USA) was used as the standard. MDA contents are expressed as nanomols per milligram of protein.

SOD activity was determined using the method of Beauchamp and Fridovich.[15] To 100 μl of supernatant, 2.85 μl of phosphate buffer 0.1 M (pH 8.4) and 50 μl of pyrogallol 7.5 mM were added, and absorbance was measured at 420 nm for 3 min at 30-s intervals. SOD levels are expressed as units per milli-gram of protein.

CAT activity was estimated using the method described by Johansson and Borg.[16] To 50 μl of supernatant, 1.0 μl of phosphate buffer 50 mM (pH 7.0) and 0.1 μl of hydrogen peroxide 30 mM were added and the decrease in absorbance at 240 nm was measured every 5 s for 30 s. CAT contents are expressed as units per milligram of protein.

Apoptosis Assay   The apoptosis assay was carried out following the method described by Pigault *et al*.[17] After 2-h preincubation with WYF (5, 50, and 500 μg/ml), the cells were cultured with Aβ_{25-35} 25 μM for 24 h. The cells were centrifugated at 1000 g for 5 min at 4°C and annexin V-FITC 10 μl and binding buffer 500 μl were added and incubated for 30 min at 4°C. PI 5 μl was then added for 5 min before the flow cytometric analysis (Becton Dickinson, USA). Ten thousand live cells were analyzed per sample.

Intracellular Concentration of Ca^{2+} ([Ca^{2+}]i) Assay   The intracellular concentration of Ca^{2+} was determined as described by Merritt *et al*.[18] After 2-h preincubation with WYF (5, 50, and 500 μg/ml) and HupA (20 μg/ml), the cells were cultured with Aβ_{25-35} 25 μM for 24 h. The cells were loaded in RPMI-1640 medium containing Fluo-3/AM 5 μM for 1 h at 37°C. After the Fluo-3/AM had been removed, the cells were incubated for 30 min in Hanks-HEPES buffer at 37°C and subsequently measured with a laser scanning confocal microscope (Leica, NT) at intervals of 5 s for 5 min.

Protein Content   Protein concentration was determined using the Bradford method.[19]

Statistical Analysis   All results are expressed as mean$\pm$S.D. Statistical significance in the MWM test and probe trial was analyzed using two-way analysis of variance (ANOVA). Student's *t*-test was used for the analysis of significance. One-way ANOVA followed by the Dunnett test was used for multiple comparisons. Values of $p < 0.05$ were considered to represent statistically significant differences.

RESULTS

In Vivo Experiments

Memory Acquisition in the MWM   In the Aβ_{25-35}-induced memory acquisition assay, the Aβ_{25-35}-treated rats increased the escape latency and path length in finding the platform ($p < 0.01$), while treatment with WYF (50 and 500 mg/kg) prevented the prolongation of the latency and path length of escap-
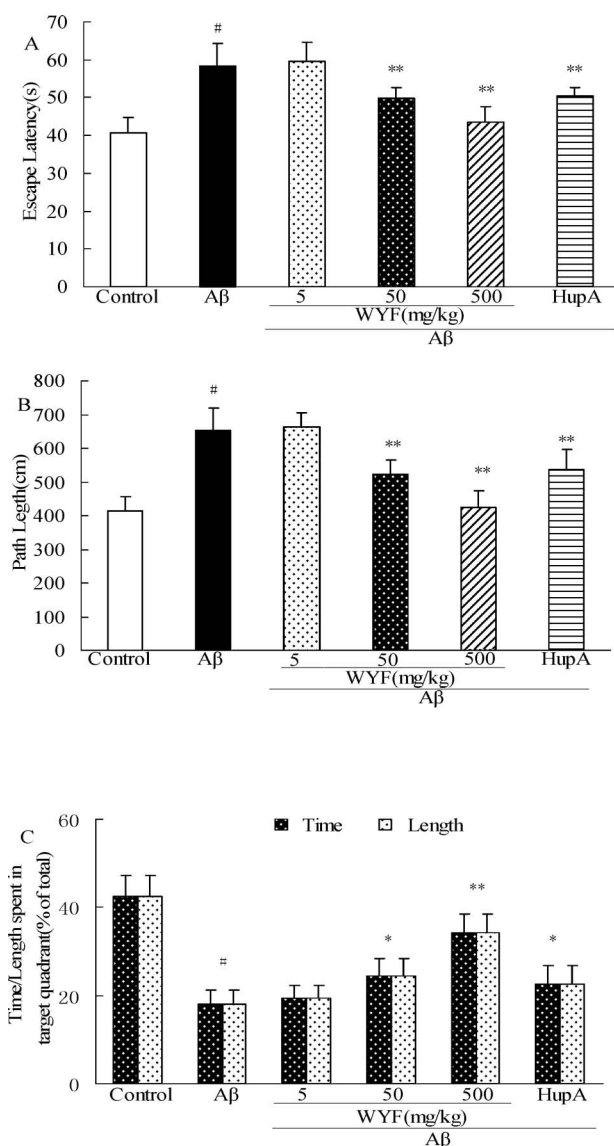
Fig. 1. Effects of WYF on $A\beta_{25-35}$-induced Memory Acquisition and Memory Retention Injury in MWM Tests and Probe Trials ($n=10$)

After receiving an i.c.v injection of either physiologic saline or $A\beta_{25-35}$ (10 mM) rats were orally administered WYF (5, 50, and 500 mg/kg) for 15 days prior to MWM testing and probe trial. HupA (20 mg/kg) served as a positive control. (A, B) The columns represent mean±S.D. of the escape latency (s) (A) and path length (cm) (B) of $A\beta$- and WYF-treated rats in each training block. (C) The columns show mean±S.D. percentage of time and length that was spent in target quadrant of the pool in the 60-s probe trial. $^\sharp p < 0.01$ vs. control group; $^*p < 0.05$, $^{**}p < 0.01$ vs. the $A\beta_{25-35}$ 10 mM-treated group.

ing onto the platform ($p < 0.01$) (Fig. 1 A and B).

Memory Retention in Probe Trials In the $A\beta_{25-35}$-induced memory retention assay, the percentage of time and the length of time spent in the target quadrant were significantly decreased in $A\beta_{25-35}$-treated animals as compared with the control group. The rats treated with WYF (50 and 500 mg/kg) spent a
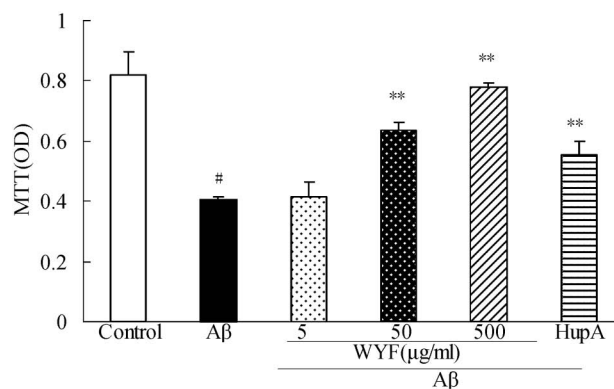

Fig. 2. Effects of WYF on Cell Viability in $A\beta_{25-35}$-injured PC12 Cells

After 2-h preincubation with WYF (5, 50, and 500 μg/ml), the cells were cultured with $A\beta_{25-35}$ 25 μM for 24 h. HupA (20 μg/ml) served as a positive control. Data are expressed as mean±S.D. ($n=3$). Statistical differences among the subgroups for each condition were determined using ANOVA, followed by the Dunnett test. $^\sharp p < 0.01$ vs. control group; $^{**}p < 0.01$ vs. $A\beta_{25-35}$ 25 μM-treated group.

greater percentage time and more length in the target quadrant than the $A\beta_{25-35}$-treated group in the probe trial ($p < 0.05$, and $p < 0.01$, rspectively) (Fig. 1C).

AChE Activity and ACh Content AChE activity was significantly elevated in the $A\beta_{25-35}$ group, and the ACh content decreased markedly ($p < 0.01$). However, after treatment with WYF (50 and 500 mg/kg), AChE activity was significantly decreased and the ACh content was significantly increased as compared with the $A\beta_{25-35}$-treated group ($p < 0.01$) (Table 1).

In Vitro Experiments

Cell Viability In the cell viability assay, cell viability was markedly decreased in the $A\beta_{25-35}$ 25 μM group. When cells were incubated with WYF (50 and 500 μg/ml) for 2 h and then exposed to $A\beta_{25-35}$, cell toxicity was significantly attenuated in a dose-dependent manner (Fig. 2).

Release of LDH and Activities of MDA, SOD, and CAT In the LDH release assay, a significant increase in LDH release was observed after 24-h exposure to $A\beta_{25-35}$ 25 μM. WYF (50 and 500 μg/ml) attenuated this increase. In the MDA, SOD, and CAT assays, treatment of PC12 cells with $A\beta_{25-35}$ 25 μM for 24 h caused an increase in the MDA level of 99.54 % and decrease in the activities of SOD and CAT by 51.27 % and 42.05 %, respectively ($p < 0.01$). However, pretreatment with WYF (50 and 500 μg/ml) for 2 h significantly reversed these effects (Table 2).

Table 1.   Effects of WYF on AchE Activity and Ach Content in Aβ_{25-35}-injured Rats $(n=10)$

Treatment	AchE (U/mg protein)	Ach (μmol/mg protein)
Control	0.10±0.03	27.78±2.94
Aβ_{25-35}	0.49±0.01[#]	17.64±2.46[#]
WYF (5 mg/kg) +Aβ_{25-35}	0.49±0.02	16.56±4.19
WYF (50 mg/kg) +Aβ_{25-35}	0.30±0.02**	23.35±3.19**
WYF (500 mg/kg) +Aβ_{25-35}	0.13±0.04**	27.20±1.68**
HupA+Aβ_{25-35}	0.28±0.01**	21.70±2.64*

All values are expressed as mean±S.D. Values were compared between the different groups using ANOVA, followed by the Dunnett test. [#] $p<0.01$ *vs.* control group ; * $p<0.05$, ** $p<0.01$ *vs.* Aβ_{25-35} 10 mM-treated group.

Table 2.   Effects of WYF on Release of LDH and Activities of MDA, SOD, and CAT in Aβ_{25-35}-injured PC12 cells

Treatment	LDH (U/L)	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Control	203.80±26.50	0.37±0.04	44.30±2.45	20.43±1.48
Aβ_{25-35}	413.76±10.50[#]	0.76±0.03[#]	23.36±1.15[#]	8.56±0.65[#]
WYF (5 μg/ml) +Aβ_{25-35}	409.56±18.85	0.73±0.04	22.13±2.56	9.23±1.65
WYF (50 μg/ml) +Aβ_{25-35}	365.43±18.43**	0.50±0.06**	30.06±1.70**	13.36±1.00**
WYF (500 μg/ml) +Aβ_{25-35}	313.96±29.35**	0.41±0.02**	39.16±2.50**	17.54±0.92**
HupA+Aβ_{25-35}	368.70±12.90**	0.53±0.11**	34.43±3.60**	14.43±1.35**

After 2-h preincubation with WYF (5, 50, and 500 μg/ml) or HupA (20 μg/ml), the cells were cultured with Aβ_{25-35} 25 μM for 24 h. Data are expressed as mean±S.D. $(n=3)$. [#] $p<0.01$ *vs.* control group ; ** $p<0.01$ *vs.* Aβ_{25-35} 25 μM-treated group.

Table 3.   Effects of WYF, TF, and TP on [Ca^{2+}]i Levels in Aβ_{25-35}-injured PC12 Cells

Treatment	[Ca^{2+}]i (nM)
Control	103.4±11.3
Aβ_{25-35}	291.7±13.7[#]
WYF (5 μg/ml) +Aβ_{25-35}	280.2±19.5
WYF (50 μg/ml) +Aβ_{25-35}	193.9±20.1**
WYF (500 μg/ml) +Aβ_{25-35}	159.5±21.2**
HupA+Aβ_{25-35}	186.3±12.3**

After 2-h preincubation with WYF (5, 50, and 500 μg/ml) or HupA (20 μg/ml), the cells were cultured with Aβ_{25-35} 25 μM for 24 h. [Ca^{2+}]i levels were measured with Fluo-3/AM. Data are expressed as mean±S.D. $(n=3)$. [#] $p<0.01$ *vs.* control group ; ** $p<0.01$ vs. Aβ_{25-35} 25 μM-treated group.

Apoptosis   In the apoptosis assay, 6.34% of cells represent apoptosis in the control group (Fig. 3A). After exposure to Aβ_{25-35} 25 μM for 24 h (Fig. 3B), apoptotic cells increased to 28.73% ($p<0.01$). Preincubation with WYF (50 and 500 μg/ml) for 2 h decreased the percentage of apoptotic cells to 20.83% and 12.73%, respectively (Fig. 3D and E) ($p<0.01$).

[Ca^{2+}]i Levels   In the [Ca^{2+}]i assay, PC12 cells that were treated with Aβ_{25-35} 25 μM for 24 h had obviously elevated [Ca^{2+}]i levels ($p<0.01$).

Pretreatment of the cells with WYF (50 and 500 μg/ml) significantly reversed the [Ca^{2+}] i increase ($p<0.01$) (Table 3).

DISCUSSION

The MWM is one of the most frequently used laboratory tools in spatial learning and memory research, typically consisting of spatial learning acquisition training in the MWM and spatial retention memory in probe trials. Spatial learning deficits are early symptoms of AD.[20] Spatial memory retention is the temporary storage and utilization of information[21] and is a function associated with the prefrontal cortex.

It has been demonstrated that there is a specific deficiency in ACh and excess of AChE in autopsy material from patients with AD.[22] The level of AChE is increased around amyloid plaques early in the process of amyloid deposition. Studies have shown that Aβ can stimulate AChE expression in embryonal carcinoma P19 cells. Recently, it has been proposed that the increase in AChE expression and activity occurs through a molecular cascade that is mediated by secreted β-amyloid precursor protein-induced microgial activation.[23] The enhancement of AChE activity
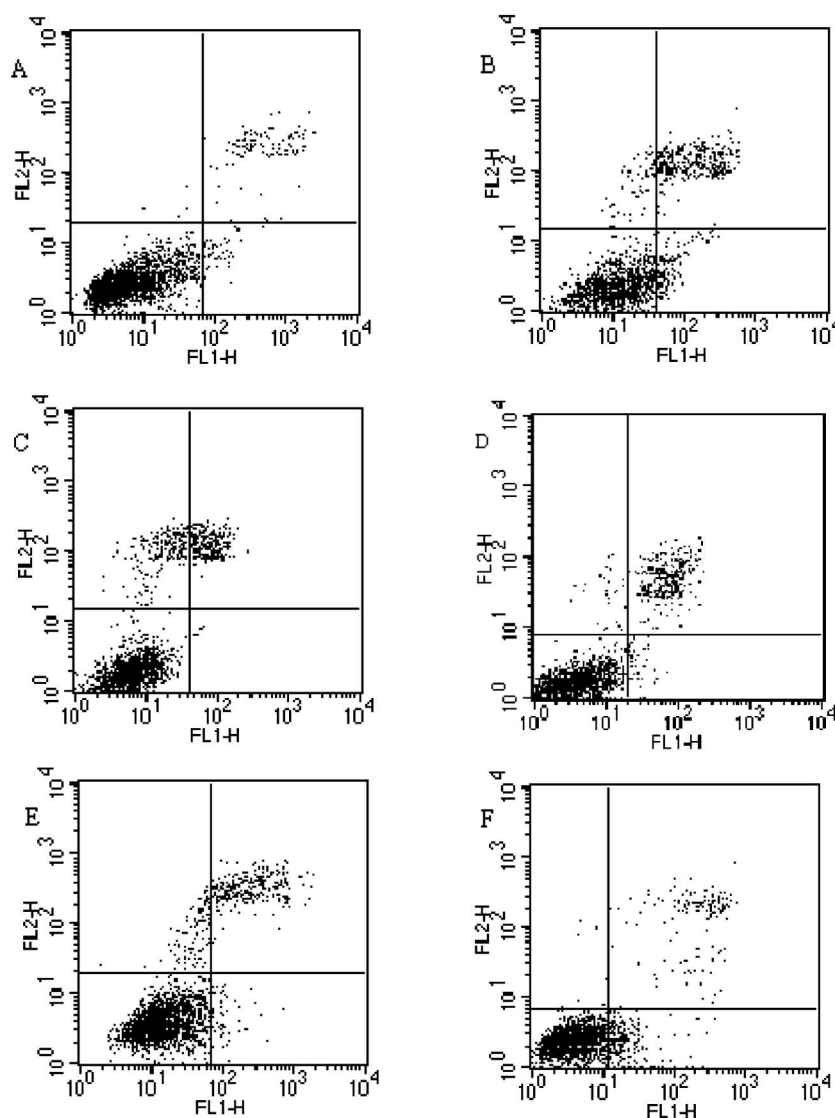
Fig. 3. Effects of WYF on $A\beta_{25-35}$-induced Apoptosis in PC12 Cells

    After 2-h preincubation with WYF (5, 50, and 500 μg/ml), the cells were cultured with $A\beta_{25-35}$ 25 μM for 24 h. HupA (20 μg/ml) served as a positive control. Data are represented as mean$\pm$S.D. ($n=3$). Statistical differences among the subgroups for each condition were determined using ANOVA, followed by the Dunnett test. A, control group; B, $A\beta_{25-35}$ 25 μM-treated group; C, WYF 5 μg/ml$+A\beta_{25-35}$ 25 μM; D, WYF 50 μg/ml$+A\beta_{25-35}$ 25 μM; E, WYF 500 μg/ml$+A\beta_{25-35}$ 25 μM; F, HupA 20 μg/ml$+A\beta_{25-35}$ 25 μM.

induced by $A\beta_{25-35}$ is also probably mediated by oxidative stress.[24] In this study, the results from AChE and Ach assays suggested that WYF notably inhibited AChE activity and increased Ach levels in the brains of rats in a concentration-dependent manner. Therefore the maintenance of acquisition and retention of spatial memory of WYF is mediated at least in part by anti-AchE effects and increasing Ach levels. The decrease in AChE activity after exposure of the cells to WYF might be a direct induction expression or the prevention of oxidative stress by WYF.

    The MTT assay was used to assess cell survival. However, herbal extracts sometimes affect absor-bance at 570 nm because of their own absorbance. We thus used a negative control and observed the cells under a microscope to determine cell survival, allowing those effects to be ignored. Oxidative stress plays a major role in cell death and AD.[25] The oxidation of membrane lipids, one of the primary events in oxidative cellular damage, can be assessed by measurement of MDA, a breakdown product of lipid peroxides.[26] On the other hand, cells are often equipped with antioxidants like SOD, CAT, and other enzymes. Inhibition of SOD and CAT activity in PC12 cells may be a consequence of decreased synthesis of SOD protein or irreversible inactivation resulting from over-

production of ROS during Aβ insult.[27] The increase in SOD and CAT activity after the exposure of the cells to WYF might be a direct induction expression or an interaction of WYF with nicotinic receptors.[28,29] Moreover, the finding that WYF reduced LDH release illustrated that WYF stabilized membrane structures and improved membrane integrity. These findings were consistent with observations from *in vivo* experiments.

ROS readily damage biological molecules, ultimately leading to apoptotic or necrotic cell death.[30] Our results suggest that WYF protects against Aβ_{25-35}-induced apoptosis *in vitro*, probably because WYF inhibits free radical formation and the propagation of free radical reactions by chelating transition metal ions. Recently, many reports have indicated that a decrease in elevations of lipid peroxidation[31] and $[Ca^{2+}]i$[32] reduced Aβ-triggered apoptosis. Pretreatment with WYF blocked the Aβ_{25-35}-induced increase in $[Ca^{2+}]i$, probably by inhibiting Ca^{2+}-activated pathways.

AD is a chronic disease requiring long-term drug administration for prevention. The currently available drugs for dementia, *e.g.*, acetylcholinesterase inhibitors, exert only a temporary effect on memory dysfunction and have serious side effects.[33] Chinese herbal medicines have been used for thousands of years in China and other Asian countries. Most prescriptions consist of a combination of several drugs, and combination therapy is a fundamental principle of Chinese medicine. The combination of multiple drugs in complex formulations is thought to maximize therapeutic efficacy by facilitating synergistic actions of the drugs and ameliorating or preventing potential adverse effects at the same time as targeting one or several pathophysiologic mechanisms.[34]

In summary, the present study demonstrated that WYF has a protective effect in *in vivo* and *in vitro* models. WYF may be used for the treatment of AD in future.

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