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Original Article

Effects of *Woo-Gui-Um* on Aβ Toxicity and Memory Dysfunction in Mice

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Objectives : Alzheimer's disease (AD) is characterized by neuronal loss and extracellular senile plaque. Moreover, the cellular actions of β-amyloid (Aβ) play a causative role in the pathogenesis of AD. This study was designed to determine whether *Woo-Gui-Um*, a commonly used Korean herbal medicine, has the ability to protect cortical and hippocampal neurons against $A\beta_{25-35}$ neurotoxicity
Methods : In the present study, the authors investigated the preventative effects of the water extract *of Woo-Gui-Um* in a mouse model of AD. Memory impairment was induced by intraventricularly (i.c.v.) injecting $A\beta_{25-35}$ peptides into mice. *Woo-Gui-Um* extract was then administered orally (p.o.) for 14 days. In addition, $A\beta_{25-35}$ toxicity on the hippocampus was assessed immunohistochemically, by staining for Tau, MAP2, TUNEL, and Bax, and by performing an *in vitro* study in PC12 cells.
Results : *Woo-Gui-Um* extract had an effect to improve learning ability and memory score in the water maze task. *Woo-Gui-Um* extract had significant neuroprotective effects *in vivo* against oxidative damage and apoptotic cell death of hippocampal neurons caused by i.c.v. $A\beta_{25-35}$. In addition, *Woo-Gui-Um* extract was found to have a protective effect on $A\beta_{25-35}$-induced apoptosis, and to promote neurite outgrowth of nerve growth factor (NGF)-differentiated PC12 cells.
Conclusions : These results suggest that *Woo-Gui-Um* extract reduces memory impairment and Alzheimer's dementia via an anti-apoptotic effect and by regulating Tau and MAP2 in the hippocampus.

Key Words : *Woo-Gui-Um*, learning and memory, $A\beta_{25-35}$, Tau, MAP2, Bax

Introduction

Aging and a number of age-related degenerative conditions in the central nervous system share the symptoms of learning and memory dysfunction. Alzheimer's disease (AD) is the most common cause of progressive intellectual failure in the elderly. Neuro-degenerative diseases are a varied assortment of central nervous system disorders characterized by the progressive loss of neural tissues[1)]. These disorders are incurable because neurons of the central nervous system cannot regenerate after cell death or damage, although current advances in stem cell research may promote neuro-regeneration or allow neuron replacement, as has been suggested by several recent studies[1)].

The i.c.v. injection of Aβ into mice provides a model of AD progression[2)]. This progression is

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accompanied by learning and memory impairments in addition to biochemical changes and neuronal degeneration. Furthermore, it is widely believed that the cellular actions of Aβ are responsible for the neuronal cell loss observed in AD, and that they play a causal role in the pathogenesis of AD. A number of studies have shown that high concentrations of Aβ are toxic and damaging biological macromolecules. Moreover, aggregated Aβ and its active fragments have been shown to have obvious neurotoxic effects[3], and intracerebral injections of Aβ or core proteins from senile plaques of the AD brain induce neuronal loss and memory impairment in mice. Therefore, mice with lesions induced by A β may represent a suitable animal model of the pathogenic mechanism leading to AD.

These effects of Aβ have been localized to amino acid residues 25-35 of the full-length peptide, and in particular the Aβ peptide fragment 25-35 ($A\beta_{25-35}$) has been shown to be directly toxic to neurons, and to increase the neurons to other insults. However, although the neurotoxicity of Aβ has been established, its precise role in the development of memory loss in AD has not been fully understood.

Neuronal cytoskeletal proteins, such as neurofilaments and microtubule-associated proteins, are disrupted in cerebral ischemia. Furthermore, microtubule-associated protein 2 (MAP2), a constituent of microtubules, is known to be a sensitive marker of ischemia[4]. Immunohistochemical studies have revealed that loss of MAP2 immunoreactivity precedes neuronal morphological changes in the hippocampus[4]. After transient ischemia, elevated glutamate levels can induce intracellular calcium accumulation[5], which activates a number of calcium-dependent enzymes that contribute to cytoskeletal alterations[6]. Furthermore, in addition to ischemic injury, abnormal MAP2 expression has been reported in the hippocampus of the AD brain[7].

The Tau protein family is a group of neuronal microtubule-associated proteins that are primarily located in neuronal axons. Because of alternative splicing of Tau mRNA, there are six isoforms that possess 352-441 amino acids and have molecular weights between 50 and 65 kDa. By binding to tubulin in axonal microtubules, Tau promotes microtubule assembly and stability[8], which is important for axonal function and transport. It has been almost 30 years since Tau was found to be a heat stable protein that facilitates *in vitro* microtubule assembly[9]. However, subsequent studies have demonstrated that Tau is a phosphoprotein, and that phosphorylation negatively regulates its ability to stimulate microtubule assembly[10].

Woo-Gui-Um is a commonly used Korean herbal medicine, and usually is used to improve renal function. Since it is believed that kidney and brain functions are closely related in Korean medicine, *Woo-Gui-Um* is also often used to treat brain-related diseases.

The present study was designed to determine whether a microinjection of $A\beta_{25-35}$ into mouse hippocampus induces learning and memory dysfunction, and further, to determine whether *Woo-Gui-Um* has the ability to protect cortical and hippocampal neurons against $A\beta_{25-35}$ neurotoxicity. For this purpose, we investigated changes in the expressions of Tau, MAP2, and BAX and in TUNEL staining induced by *Woo-Gui-Um* administered after Aβ. In addition, we examined the effects of *Woo-Gui-Um* on $A\beta_{25-35}$ induced apoptosis and on PC12 cell neurite outgrowth after pre-treating cells with *Woo-Gui-Um* extract and NGF.

Materials and Methods

1. Preparation and administration of Woo-Gui-Um

The ingredients of *Woo-Gui-Um* are shown in Table. 1. *Woo-Gui-Um* (525g) was boiled in distilled water (2L) for 2h, and the filtrate so obtained was evaporated under reduced pressure to yield an aqueous extract, which when lyophilized yielded 38.9g of *Woo-Gui-Um*.

Table 1. Constituents of Woo-Gui-Um

Herb medicine	Scientific name	Medical Use	Dosage(g)/Chup*
Aconiti Tuber	Aconitum carmichieli Debx.	a lateral root	3.75
Cinnamomi Corfex Spissus	Cinnamomum cassia Bl.	a bark	3.75
Rehmammiae Rakix Vapratum	Rehmannia glutinosa Libosch· f. hueichingensis(Chao et Schih) Hsiao	repeatedly heat with steam rootstock	37.5
Corni Fructus	Cornus offcinalis Sieb. et Zucc.	a sarcocarp	3.75
Dioscoreae Radix	Dioscorea batatas Decne.	a lump root	7.5
Lycii Fructrs	Lycium chinense Mill.	ripened fruit	7.5
Eucommiae Cortex	Eucommia ulmoides Oliv.	a dry bark	7.5
Glycyrrhizae Radix	Glycyrrhiza uralensis Fisch.	burn a root or rootstock	3.75

* (Chup): A standard unit of prescription (a dose).

2. Animals

Male BALB/c mice weighing 25-30 g (11 weeks old) were maintained in a climate-controlled room at 22±1˚C under a 12 h light/dark cycle with free access to food and water. The study was approved by the Institutional Animal Care and Use Committee of Kyung Hee University and all procedures were conducted in accordance with the U.S. National Institutes of Health guidelines.

3. Injection of $A\beta_{25-35}$

Before the injection, $A\beta_{25-35}$ (A4559, Sigma-Aldrich, U.S.A) was aggregated by incubating it at 37+˚C for 72 h. Aggregated $A\beta_{25-35}$ was then injected (i.c.v.) into mice. Briefly, a 26-gauge stainless-steel needle was inserted unilaterally 1 mm to the right of the midline point, equidistant from each eye, at an equal distance between the eyes and ears and perpendicularly to the plane of the skull. $A\beta_{25-35}$ or sterile normal saline (5 μL) was delivered gradually over 15 s.

4. Administration of *Woo-Gui-Um*

Mice that had been injected with $A\beta_{25-35}$ were randomly divided into two groups. A solution of *Woo-Gui-Um* (1.3mg/10g) in normal saline or just normal saline were given orally once daily for 14 days after surgery.

5. Place Learning in the Water-maze

Morris water maze testing was started at 14 days after surgery. The experimental apparatus consisted of a circular water tank (diameter=90 cm) filled to a depth of 20.5 cm with water maintained at 23+1˚C. The water was opacified by adding milk. A platform (diameter=10cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. For each trial, one mouse was placed in the pool at one starting position, and allowed to swim freely until it had found the platform. The time required to find the hidden platform was recorded. Mice that found the platform were allowed to remain on the platform for 10s and were then returned to their home cages. If a mouse did not find the platform within 60s, it was gently guided to the platform by the experimenter, where it remained for 10s. Escape latency (EL) was defined as the time required for a mouse to find the platform. Each group of animals was tested in this manner for a total of 5 trials/animal on day 1, and 3 trials/animal on day 2.

On day 3, each mouse was allowed to swim

freely for 60s, and their swimming tracks were recorded over this 60s period using SMART (ver. 2.5, Panl Ab, Spain).

Statistic comparisons of the water maze test performance of Aβ-treated and saline treated mice were performed using one-way analysis of variance (ANOVA).

6. Memory retention test

The Morris water maze was used to investigate how spatial memory retention was altered in three groups, i.e., the sham operated group (group treated with saline), Aβ injected group (Aβ), and group injected with Aβ injection and treated with *Woo-Gui-Um* (WGU). The swimming field was divided into 5 zones and different points were allocated to zones according to the distance from the platform. (Zone 1 : -1, Zone 2 : +1, Zone 3 : +8, Zone 4 : +5, Target (Platform) : +10, Fig. 1)

To get the memory retention points of each mice, each mouse was allowed to swim freely for 60s at day 3. The total points of each mouse were calculated in summation of multiplying swimming times by different points of each zone.

7. Tissue preparation

16 days after surgery, animals were anesthetized with sodium pentobarbital (25 ㎎/㎏, i.m) and then transcardially perfused with 10% formalin in 0.1 M phosphate buffer saline, pH 7.4 (PBS). Brains were immediately removed and fixed in the same fixative solution for 24 h. They were then embedded in paraffin and 8 ㎛ thick sections were cut and mounted on poly-L-lysine coated slides.

8. Immunohistochemistry

Immunohistochemistry was carried using the avidin-biotin-peroxidase method (Vectastatin ABC kit, Vector Labs). In brief, 8 ㎛ deparaffinized sections were incubated with 0.3% hydrogen peroxide and methanol for 20 min to reduce nonspecific staining and then boiled in 10 mM citrate buffer (pH 6.0) in microwave oven for 10 min to retrieve antigens. Mouse anti-Bax (B-9, Santa Cruz, CA), mouse anti-Tau (MN1000, Pierce endogen, USA) and anti-MAP2 antibodies in 0.05M Tris-buffered solution (TBS) were added to slides and incubated overnight at 4℃. The sections were then incubated for 1 h with biotinylated and anti-mouse IgG antibody (Vector) and finally, with ABC complex at a dilution of 1:100 for 1h. Peroxidase was visualized using 0.05% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in TBS. Specificity of the immunoreaction was tested by incubating sections without primarily antibody.

9. Measurement of DNA fragmentation

Cleaved DNA was identified using a modified end-labeling technique, originally described by Gavrieli *et al*. (Apoptag Kit, Intergen, USA). Deparaffinized sections were permeabilized with proteinase K (20 ㎍/ml) for 15 min at room temperature and then washed four times with deionized water for 20 min. Endogenous peroxidase was quenched with 3% (v/v) hydrogen peroxide in PBS for 5 min and tissues were rinsed in PBS (2×5min). After equilibration in buffer, sections were incubated with terminal deoxynucleotidyl transferase (TdT) containing digoxigenin-dUTP (0.3 e.u./ml) at 37 ℃ for 1 h in a humidified chamber. The TdT reaction was stopped by immersing sections in Stop/Wash buffer for 10 min, and then washed with PBS (3×15 min). Anti-digoxigenin peroxidase was added to each tissue section and sections were incubated for an additional 30 min at room temperature. Staining was developed using diaminobenzidine.

10. $A\beta_{25-35}$ and *Woo-Gui-Um* treatment and PC12 cell viability assays

PC12 cells (a rat pheochromocytoma cell-line) were maintained in DMEM supplemented with 5% fetal bovine serum (FBS) and 10% horse serum (Invitrogen, Gaithberg, MD). Cells were plated on PLL-coated 96-well plates and chamber slide glass (Lab-Tek, Nunc International, USA) at a density of

4×10^4/ ml and then maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were allowed cultured in prior to each treatment.

Amyloid β-peptide fragment 25-35 ($A\beta_{25-35}$, Sigma) was dissolved in distilled water at 1 mM, and stored at -20˚C. *Woo-Gui-Um* was dissolved and diluted with culture grade phosphate-buffered saline (PBS). The cells were placed in serum free medium and preincubated with *Woo-Gui-Um* (0.5, 5, 50 ㎍/ml) for 1hr before adding $A\beta_{25-35}$ (200 μM/ml). After 24hr of exposure to $A\beta_{25-35}$, cell viabilities were evaluated by MTT reduction. Briefly, MTT solution in PBS was added for 2 hr. Media were then removed and cells were dissolved in DMSO. [missing term] was quantified by measuring absorbance at 570nm using an ELISA Reader (SOFT max PRO 4.3LS).

11. Immunofluorescence Staining of Woo-Gui-Um and/or NGF-treated PC12 cell

To induce neuronal differentiation, PC12 cells were treated with NGF (100 ng/ml, R&D System, USA) and *Woo-Gui-Um* (0.5 ㎍/ml) in serum free DMEM medium. Medium containing NGF or *Woo-Gui-Um* were changed on days 0, 2, and 4. The next day, cells were rinsed with PBS and fixed in 4% paraformaldehyde. The cells were then washed with PBS, permeabilized in 0.2% Triton X-100 in PBS, and blocked in 10% horse serum containing 1% bovine serum albumin (BSA). After being incubated with anti-MAP2 (1:100, Sigma, USA) and subsequently with anti-mouse Cy3 (1:100, Jackson ImmunoResearch, USA), cells were observed under a confocal laser-scanning microscope (Carl Zeiss, LSM 510 META, Germany)

12. Statistical analysis.

All data are presented as means + standard errors. Statistical analysis was performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one-way ANOVA followed by Duncan's multiple-range test. Statistical significance was accepted for p values of <0.05.

Results

1. Effect of *Woo-Gui-Um* on place learning in the water-maze test

$A\beta_{25-35}$ or normal saline were administered i.c.v. 14 days before the first training day. A solution of *Woo-Gui-Um* and normal saline were given orally once daily for 14 days after surgery. Escape latency of the sham group decreased over the course of acquisition training on days 1 and 2. Between individual trials, escape latency significantly declined from trial 1 to trial 2 (on day 1) and from trial 6 to trial 7 (on day 2). For the $A\beta_{25-35}$-treated group, escape latency did not significantly decrease over the course of acquisition training. Furthermore, in the $A\beta_{25-35}$-treated group, escape latency during trial 6 (the first trial on day 2, (54.63+4s)) was similar to that of trial 1 (the first trial of day 1 (53.1+7.4s). On the other hand, escape latency in the *Woo-Gui-Um* treated group decreased in a manner similar to

Table 2. Mean Escape Latencies in the Study Groups

	Day 1				Day 2			
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8
Sham	51.0+5.5	36.5+7.2	18.1+5.6	15.5+4.0	12.0+7.8	29.4+4.3	17.4+5.0	16.0+3.2
Aβ inj.	53.1+7.4	48.4+2.7	45.5+7.1	38.5+4.7	36.1+4.7	54.6+4.0	46.5+5.1	46.0+3.8
WGU treat.	53.3+10.1	38.1+4.9*	28.1+6.7*	22.0+2.6*	21.6+4.1*	36.1+8.4*	23.3+8.0*	20.0+7.8*

Sham: group treated with saline
Aβ inj.: Aβ-injected group
WGU treat.: group injected with Aβ and treated with *Woo-Gui-Um*
Data represent means + standard errors. *: $P < 0.01$ vs. the Aβ group.

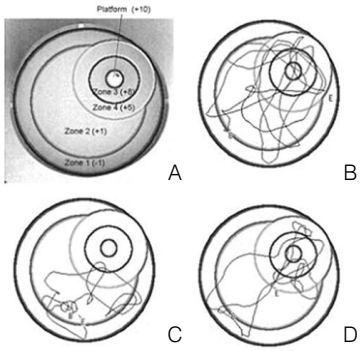

Fig. 1. Zones about the submerged platform (A) and representative swimming paths of mice from starting positions (B-D). Different points were allocated to the zones; Zone 1 : -1, Zone 2 : +1, Zone 3 : +8, Zone 4 : +5, Target (Platform) : +10. Sham-treated animal (B) showing typical swimming pathway after 2 days of training. Aβ-treated animal (C) showing a loss of directional bearing. Woo-Gui-Um plus Aβ-treated animal (D) showing directional recovery.

that observed in the sham group over the course of acquisition training. (Table. 2)

2. Effect of *Woo-Gui-Um* on memory retention test results

Aβ-injected mice swam randomly rather than directly to the hidden platform, and did not find the platform as quickly as mice in the sham-operated group. In comparison mice in the *Woo-Gui-Um* group found the platform comparatively easily (Fig. 1). In terms of memory retention points, Aβ-injected mice scored lower than sham-operated mice and *Woo-Gui-Um* treated mice.

3. Immunohistochemical detection of Bax

Bax expression was scarcely observed in the cytoplasm of CA1 neurons in the normal mouse group. However, Bax immunoreactivity was obvious in CA1 neurons at 14 days after surgery (Fig. 2; C,D).

Furthermore, the immunoreactivity of Bax was obviously lower in CA1 neurons of the *Woo-Gui-Um* group than in those of the saline treated group (Fig. 2; E,F).

4. TUNEL staining

TUNEL-positive neurons were not observed in the sham group, whereas the Aβ injected group showed positive TUNEL staining (Fig. 3), and the

Table 3. Memory Retention Points Awarded to the Study Groups

	Sham	Aβ inj.	WGU treat
Zone 1	9.8	11.2	13.4
Zone 2	31.2	38.1	28.5
Zone 3	7.8	2.8	6.5
Zone 4	9.7	5.5	9.9
Platform	0.6	0.1	0.5
Total	138.7±12.2	76.6±4.1	121.2±16.6*
N	8	8	7

On day 3 after two-day acquisition sessions, mice were allowed to swim freely for 60s. Total points for each mouse were calculated by summing the products of swimming times and zone points. Data represent means + standard errors. *: $P < 0.01$ vs. the Aβ inj.

Table 4. Bax, TUNEL, MAP2 and Tau Expressional Changes in the Hippocampus

Groups	Immuno-reactivity			
	Bax	TUNEL	MAP2	Tau
Sham	-	-	++	-
Aβ inj.	++	+	±	+
WGU treat.	+	±	+	±

Sham: group treated with saline
Aβ inj.: Aβ-injected group
WGU treat.: Aβ injected and Woo-Gui-Um treated
Immuno-densities are demonstrated as ±, weak; +, mild; and ++, moderate.

Woo-Gui-Um treated group slight TUNEL staining.

5. Immunohistochemical detection of MAP II and Tau

Representative photographs of MAP2 immunostaining in the hippocampal CA1 region are shown in Fig. 9. Pyramidal neurons with MAP2 immunoreactivity were easily detected in this sector. In the sham-operated group, the bodies and fibers of CA1 pyramidal neurons were intensely stained with immunopositive processes. In particular, dendrites and axons of hippocampal CA1 neurons were more markedly affected than cell bodies. In the Aβ-injected group a decrease in MAP2 immunoreactivity was observed in CA1. Dendrites and axons of CA1 pyramidal neurons also became irregular and fragmented

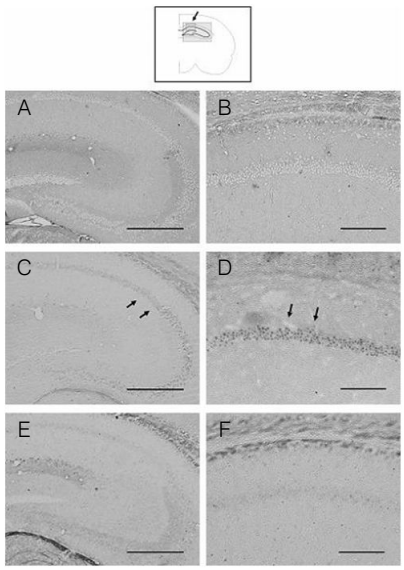

Fig. 2. Immunohistochemical analysis of Bax protein in CA1 hippocampal sections from the sham (A, B), Aβ (C, D), and Woo-Gui-Um (E, F) groups at 14 days after surgery. B, D, F show magnified images of the CA1 area in A, C, E. (A, C, E; X100, B, D, F; X200). Scale bar A, C, E = 500 ㎛ ; B, D, F = 200 ㎛.

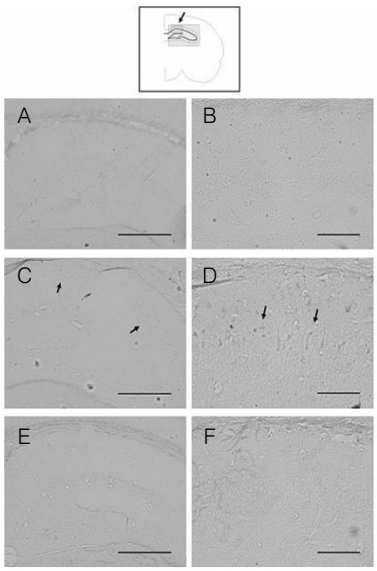

Fig. 3. Terminal transferase dUTP nick-end labeling (TUNEL) staining of hippocampal CA1 pyramidal neurons in the sham (A, B), Aβ (C, D) and WGU (E, F) groups. No significant inter-group differences were observed. (A, C, E; X100, B, D, F; X200). Scale bar A, C, E = 500 ㎛ ; B, D, F = 200 ㎛.

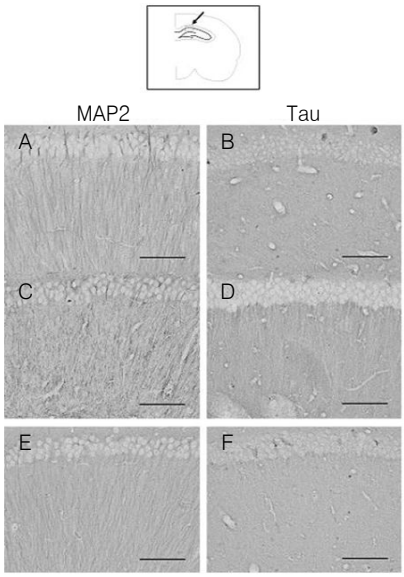

Fig. 4. Immunohistochemical analysis of MAP2 (A, C, E) and Tau (B, D, F) proteins in sections from the sham (A, B), Aβ (C, D), and WGU (E, F) groups. X400. Scale bar = 100 ㎛

in places. However, MAP2 immunoreactivity and neuron fiber feature had recovered in CA1 in the *Woo-Gui-Um* group (Fig. 4.). To determine the effect of *Woo-Gui-Um* administration on Tau pathology, we examined the phosphorylation and aggregation state of Tau in the three study groups. Tau phosphorylation and aggregation were more enhanced around hippocampal CA1 neurons in the Aβ group than in the sham group, whereas the *Woo-Gui-Um* treated group showed less Tau immunoreactivity than the Aβ group (Fig. 4.).

6. Effects of *Woo-Gui-Um* on $A\beta_{25-35}$ induced apoptosis

Losses of cell viabilities in PC12 cell cultures were determined by measuring reductions in MTT

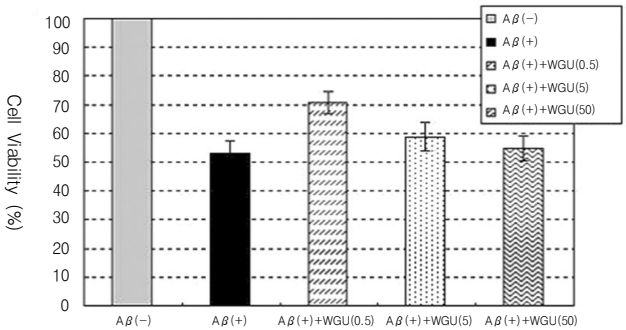

Fig. 5. Protective effects of Woo-Gui-Um on Aβ25-35-induced apoptosis. PC12 cells were pre-treated with Woo-Gui-Um (0.5, 5, 50㎍/ml) for 1 hr before Aβ25-35 (200μM/ml) was added. 24 hr after exposure to Aβ25-35, cell viabilities were evaluated using MTT assays.

activity. Treatment with $A\beta_{25-35}$ resulted in a decrease in cell viability versus untreated controls, and preincubation in *Woo-Gui-Um* extract for 1 h protected cells from $A\beta_{25-35}$ toxicity. Moreover, cell viability decreased in a dose-dependent manner after $A\beta_{25-35}$ treatment (Fig. 5). Our results showed that the protective effect of *Woo-Gui-Um* was greatest at 0.5 ㎍/㎖.

7. Observations of the divisions of PC12 neurites

The effects of *Woo-Gui-Um* extract on nerve growth factor (NGF)-induced PC12 cell differentiation were examined, and in particular, we observed neurites division. To induce differentiation, cells were treated with NGF (100 ng/ml) and *Woo-Gui-Um* extract (0.5 ㎍/ml) in serum free DMEM. NGF and *Woo-Gui-Um* extract containing media were changed on days 0, 2, and 4. On day 5 cells were incubated with anti-MAP2 and then with anti-mouse Cy3, and observed under a confocal laser-scanning microscope.

Naive PC12 cells did not show any basal neurite outgrowth (Fig. 6A). However, after treatment with NGF alone, almost all cells showed neurite outgrowth (Fig. 6B). Moreover, after incubation with *Woo-Gui-Um* extract, about 43% of the cells showed neurite outgrowth (Fig. 6C). However, in presence of NGF plus *Woo-Gui-Um* extract, neurite outgrowths were observed on all PC12 cells, and their lengths were greater than those of cells treated with NGF alone (Fig. 6D).

Discussion

Woo-Gui-Um is a well-known Oriental medicine that is usually prescribed to improve renal functions and to treat brain-related diseases. AD is characterized by neuronal loss and by the formation of extracellular senile plaques containing amyloid-β peptide (Aβ), a 39-43 amino acid peptide derived from amyloid precursor protein (APP)[11]. Neuronal loss occurs via apoptosis, a type of cell death with distinct morphological and biochemical characteristics, and neuronal apoptosis is one of the pathological features of AD[11]. Furthermore, Aβ has been shown to be neurotoxic *in vitro*[12] and *in vivo*[13], and Aβ -induced cell death exhibits the typical features of apoptosis, i.e., neurite beading, membrane blebbing, chromatin condensation, and DNA fragmentation[14].

In the present study, using the PC12 cell line, which has been used as a model for neuronal functional studies, we investigated the effects of

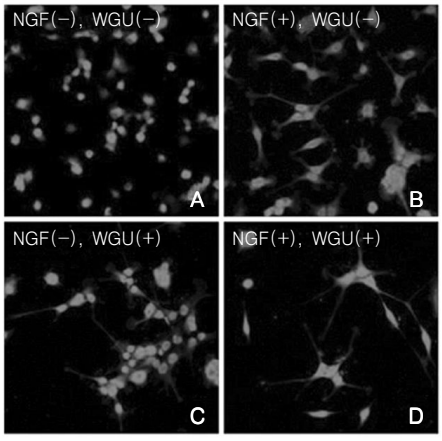

Fig. 6. Differential neurite outgrowth responses of PC12 cells to treatment with NGF (100 ng/ml) and Woo-Gui-Um extract (0.5 ㎍/ml). The images show immunofluorescence staining of neuronal PC12 cells treated with NGF and/or Woo-Gui-Um extract. Cells were observed by confocal laser-scanning microscopy.

Woo-Gui-Um extract on neuronal apoptosis induced by $A\beta_{25-35}$. Experiments were aimed at determining whether *Woo-Gui-Um* extract can protect neurons from Aβ-induced apoptosis. The results obtained indicate that *Woo-Gui-Um* extract does indeed have neuroprotective effects on the neuronal apoptosis induced by $A\beta_{25-35}$.

Nerve growth factor (NGF) has profound effects on the survival and differentiation of PC12 cells. On exposure to NGF, PC12 cells extend neuron-like processes and differentiate into sympathetic neuron-like cells. In the present study, it was observed that *Woo-Gui-Um* extract enhanced NGF-stimulated neurite outgrowth in serum-starved PC12 cells, and that PC12 cells not stimulated by NGF showed no basal neurite outgrowth. When treated with NGF alone, almost all PC12 cells showed neurite outgrowths, and in the presence of NGF and *Woo-Gui-Um* extract these neurite outgrowths were enhanced for all cells, and furthermore, their lengths were greater than those of cells treated with NGF alone.

Many studies have been undertaken to determine whether β-amyloid has a neurotoxic effect, since it was reported that Aβ and its aggregates have toxic effects on cultured neurons *in vitro*. Moreover, intracerebral injections of β-amyloid or core protein from senile plaques from Alzheimer's brain are known to induce neuronal degeneration characterized by the induction of argyrophilic neurophil thread formation and AD-specific antigen production in rodents. More recently, similar degenerative morphological changes have been reported for primate neurons after β-amyloid injection. Furthermore, β-amyloid injection also produced amnestic effects on mice in behavioral tests[2]. In the present study, we injected Aβ in the same manner as has been used in AD models.

In the present study, long-term memory was examined using the place learning procedure in the water-maze, between 10 and 14 days after the i.c.v. administration of aged $A\beta_{25-35}$. It was found that escape latency in the saline treated group decreased over the course of acquisition training, and that between trials, a significant decline in escape latency was observed between trial 1 on day 1 and trials 4 and 5 on day 1, and between trial 1 and each trial performed on day 2 ($P<0.01$ each). However, in the $A\beta_{25-35}$-treated group, escape latency did not significantly decrease over the course of acquisition training, and the $A\beta_{25-35}$-treated group had significantly greater escape latencies than the control group during the two last trials on each day. These findings suggest that treated with β-amyloid peptide, 10 days previously, produced a marked learning impairment[2]. In a previous report, it was reported that 10-month-old SD rats injected bilaterally with $A\beta_{25-35}$ ($20\mu g$) or vehicle into the hippocampus, had higher mean latencies, swimming paths (distance), and starting angles in the Morris water maze test, which demonstrates that $A\beta_{25-35}$ treated rats require more time to find that the platform than non-treated controls[15]. Hu Z.H. employed the Morris water maze to investigate how spatial memory retention was altered in isoproterenol-injected rats. After pre-training animals for a week, all rats were able to swim to the hidden platform within 10 s. These rats were randomly divided into two groups and injected i.c.v. with isoproterenol or saline, respectively. Memory retention test data at 24 h after injection demonstrated that escape latencies in isoproterenol-treated rats were remarkably greater than those of non-injected controls (49.83+8.65s vs. 6.55+2.42s, $P<0.01$), while the escape latencies of saline rats were similar to those of controls (10.44+5.78s vs. 6.74+2.37s, P=0.14). Furthermore, significant differences was also observed between isoproterenol and saline-treated animals (49.83+8.65s vs. 10.44+5.78s, $P<0.01$). Furthermore, swimming pathway results showed that isoproterenol-injected rats swam randomly rather than directly to the hidden platform like saline-treated controls or the pre-surgery controls[16]. In the present study we also employed the Morris maze test to examine whether *Woo-Gui-Um* affects the memory impairment induced by the i.c.v. administration of aggregated $A\beta_{25-35}$ in mice as a first step toward evaluating its potential value for

the treatment of Alzheimer's disease. The results obtained showed that, at the last trial (trial 8), escape latency to find the hidden platform for the A β_{25-35} group was remarkably greater than that of sham group (46+3.8s vs. 16+3.2s, $P<0.01$). However, escape latency in the *Woo-Gui-Um* group decreased in the same manner as that observed in the sham group over the course of acquisition training. The present results, like previous study, show that an i.c.v. injection of Aβ_{25-35} into mice induces learning impairment by the Morris water maze test and that treatment with *Woo-Gui-Um* has a protective effect on the memory impairment induced by the i.c.v. administration of aggregated Aβ_{25-35} in mice.

Microtubules are composed for polymerized tubulin dimers composed of α- and β-subunits and various microtubule-associated proteins (MAPs). MAP Tau, MAP1 (A/B), and MAP2 are major microtubule-associated proteins in normal mature neurons. These three MAPs apparently perform similar functions, i.e., they promote the assembly and stability of microtubules. This excessive redundancy in biology, i.e., having three different proteins to maintain the microtubule network in a neuron, is probably due to the essential requirement of microtubules for axoplasmic flow, which, in turn, is critically required for neuronal activity. Thus, neurons have the capacity to compensate for the loss of function of one MAP by utilizing the other two MAPs. Both Tau and MAP2 transgenic knockout mice show apparently normal development to adulthood, whereas Tau plus MAP2 and MAP2 plus MAP1B transgenic double knockout mice show defects in axonal elongation and neuronal migration[17]. MAP2 is present in dendrites, nerve cell bodies, and axons[18], and is a well-known marker of neuronal damage associated with central nervous system (CNS) injury[19].

In the present study, MAP2 immunoreactive pyramidal neurons are present in the hippocampal CA1 region. In the sham operated group, the bodies and fibers of CA1 pyramidal neurons were intensely stained with evident immunopositive processes, though dendrites and axons were less markedly stained. However, in the Aβ group, MAP2 immunoreactivity was reduced in CA1, and the dendrites and axons of CA1 pyramidal neurons were irregular and fragmented in places. On the other hand, in the *Woo-Gui-Um* treated group, MAP2 immunoreactivity and neuronal fiber features were intermediate between these two. These findings suggest that *Woo-Gui-Um* extract has protective effect against the memory impairment induced by Aβ_{25-35} because it protect cells from MAP2-induce damage.

Senile plaques result from the extracellular accumulation of Aβ peptide and the formation of amyloid deposits. Aβ is derived from β-amyloid precursor protein (APP). In cases of familial AD, mutations have been found in the APP gene, which suggests that this gene plays a central role in the etiopathogenesis of familial AD[20]. Senile plaques are diffusely and variably distributed throughout cerebral and subcortical structures, and NFTs correspond to aggregations of abnormal fibrils into PHF[21], within certain vulnerable neuronal populations. At the microscopic level, NFTs are preferentially observed in the large pyramidal cells of the hippocampus and the entorhinal cortex, and in the II-III (supragranular) and V-VI layers of association cortical areas, while primary sensory and motor cortices are relatively spared[22]. NFTs account for the synaptic degeneration or the atrophy of nerve cells following damage to synaptically-connected axons. NFTs are composed of paired helical filaments that are primarily composed of insoluble hyperphosphorylated Tau protein[23].

The most obvious pathological event that occurs during several neuro-degenerative disorders is the aggregation of Tau isoforms into intraneuronal filamentous inclusions. Until recently, it was considered that abnormal Tau protein phosphorylation was responsible for its aggregation in AD. In numerous neuro-degenerative disorders, Tau proteins aggregate into intraneuronal filamentous inclusions. In AD, their constitutive proteins are referred to as PHF-Tau proteins. The main feature of pathological Tau proteins is their ability to aggregate into the polymers

that constitute neurofibrillary lesions in AD. In addition, specific phosphorylation sites are also present on PHF-Tau, and these may be associated with the aggregation process. However, Tau aggregation is not specific to AD, and has also been described in many other neuro-degenerative disorders, though interestingly, Tau protein electrophoretic profiles are often disease-specific.

Using immunological probes specific for Tau phosphorylation sites, it is possible to investigate NFT biochemically in postmortem brain materials. A strong correlation between the immunohistochemical detection of NFT and the presence of the Tau triplet has been demonstrated, which indicates that NFT is a reliable marker of the degenerative process, and that pathological Tau proteins can be used to quantify neurofibrillary degeneration[24]. Furthermore, biochemical mappings based on immunoblotting and/or ELISA findings have been performed in several cortical areas in patients with senile dementia of the Alzheimer's type. These analyses revealed that pathological Tau triplet in all studied areas, with the exception of a few regions, such as, the primary motor and visual cortices. The level of the pathological Tau triplet was found to be higher in the cortex than in the primary sensory cortex; and highest levels were observed in the temporal neocortical and limbic areas. However, for a given brain area, Tau immunoreactivity varies on an individual basis, especially in terms of the phosphorylation of the longest Tau isoform[25].

In a study on the exacerbation of Tau pathology by chronic nicotine administration in a transgenic model of AD, the phosphorylation and aggregation states of Tau were compared for nicotine-treated and untreated transgenic mice. The hippocampus was focused upon because it is the first region to develop Tau pathology in transgenic mice. At 6 months, the first notable change in Tau immunoreactivity was observed within the somatodendritic compartment of CA1 pyramidal neurons. It was found that administering nicotine for 5 months markedly increased the somatodendritic labeling of Tau in CA1 pyramidal neurons of 6-month-old transgenic mice compared with age-matched untreated mice. Moreover, in nicotine-treated mice, virtually all CA1 neurons showed robust Tau somatodendritic immunostaining, indicating Tau mislocalization from the axonal compartment, an early event in tangle formation[26].

The present results show that the phosphorylation and aggregation of Tau are enhanced around hippocampal CA1 neurons after Aβ injection as compared with the sham-operated group. However, in *Woo-Gui-Um* and Aβ-treated animals, Tau immunoreactivity was lower than for Aβ-treated animals. Furthermore, in our *in vitro* study, after treatment with WGU extract alone about 43% of cells showed neurite outgrowth, and in presence of NGF and WGU, all PC12 cells showed neurite outgrowths, and the lengths of these neurite outgrowths were greater than those of cells treated with NGF alone.

The present study demonstrates that a single acute i.c.v. injection of an aged preparation of $A\beta_{25-35}$ into the hippocampus induces marked amnesic effects in mice. These effects were evidenced using a repetitive place learning procedure in a Morris water maze, an accepted index of spatial working memory. Moreover, *Woo-Gui-Um* administration was found to significantly reduce the detrimental effects of $A\beta_{25-35}$ on learning and memory dysfunction. Furthermore, our findings suggest that *Woo-Gui-Um* ameliorates the effects of $A\beta_{25-35}$ by regulating Tau and MAP2 at the protein level.

In conclusion, our results suggest that *Woo-Gui-Um* extract protected mice from the neuronal and memory losses induced by $A\beta_{25-35}$. These results also suggest that *Woo-Gui-Um* may have a therapeutic effect on the development of dementia due to Alzheimer's disease.

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