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Amyloid β protein (25-35)-유도 배양신경 세포독성 및 마우스기억손상에 대한 목과의 억제효과

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Inhibitory Effect of *Chaenomeles sinensis* Fruit on Amyloid β Protein (25-35)-Induced Neurotoxicity in Cultured Neurons and Memory Impairment in Mice

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ABSTRACT : The present study investigated an ethanol extract of *Chaenomeles sinensis* fruit (CSF) for possible neuroprotective effects on neurotoxicity induced by amyloid β protein (Aβ) (25-35) in cultured rat cortical neurons and also for antidementia activity in mice. Exposure of cultured cortical neurons to 10 μM Aβ (25-35) for 36 h induced neuronal apoptotic death. At 0.1-10 ㎍/㎖, CSF inhibited neuronal death, elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), and generation of reactive oxygen species (ROS) induced by Aβ (25-35) in primary cultures of rat cortical neurons. Memory loss induced by intracerebroventricular injection of mice with 15 nmol Aβ (25-35) was inhibited by chronic treatment with CSF (10, 25 and 50 ㎎/㎏, p.o. for 7 days) as measured by a passive avoidance test. CSF (50 ㎎/㎏) inhibited the increase of cholinesterase activity in Aβ (25-35)-injected mice brain. From these results, we suggest that the antidementia effect of CSF is due to its neuroprotective effect against Aβ (25-35)-induced neurotoxicity and that CSF may have a therapeutic role for preventing the progression of Alzheimer's disease.

Key Words **:** *Chaenomeles sinensis* Fruit, Neuroprotection, Amyloid β Protein, Cultured Neurons, Memory Impairment

INTRODUCTION

Chaenomeles sinensis (Thouin) Koehne fruit (family: Rosaceae) (CSF) has been widely used as a traditional Chinese medicine to treat throat disease. It is known as an antitussive, antiflatulent and diuretic agent in folk medicine. CSF is rich in dietary fibre, organic acids and bioactive pentacyclic triterpene acids such as oleanolic acid and ursolic acid and large amounts of bioactive phenolic acids and vitamin C (Hamauzu *et al*., 2008; Ros *et al*., 2004; Thomas *et al*., 2000; Zhang *et al*., 2007). Flavonoid is one of the main phytochemical constituents in CSF and has been proven effective in preventing free radical-caused oxidative damage (Hamauzu *et al*., 2005; Zhou *et al*., 2007; Hu *et al*., 2008). Antibacterial, antiviral, antihemolytic and antipruritic activities have been reported as pharmacological activities of CSF (Osawa *et al*., 1997; Lee *et al*., 2002; Oku *et al*., 2003; Sawai *et al*., 2008). Anti-inflammatory effect of CSF was inferred based on the fact that it inhibited histamine release from rat mast cells and inhibited activation of hyaluronidase (Osawa *et al*., 1999, 2001).

Alzheimer's disease (AD) is characterized by neuronal loss and extracellular senile plaque, whose major constituent is amyloid β protein (Aβ), a 39-43 amino acid peptide derived from amyloid precursor protein (Ivins *et al*., 1999). Both *in vitro* and *in vivo* studies have reported the toxic effects of Aβ or Aβ peptide fragments suggesting an important role of Aβ in the pathogenesis of AD (Demuro *et al*., 2010). The mechanisms underlying Aβ-neurotoxicity are complex but may involve *N*-methyl-$_D$-aspartate (NMDA) receptor, a glutamate receptor subtype, modulation induced by glutamate release, sustained elevations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and oxidative stresses (Ekinci *et al*., 2000; Gray and Patel, 1995;

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Ueda *et al*., 1997).

An experimental model that mimics the progression of AD was developed using an intracerebroventricular (i.c.v.) injection of Aβ in mice (Van Dam and De Deyn, 2006). I.c.v. Injection of Aβ (25-35) in mice resulted in learning and memory deficits that were accompanied by a decrease of choline-acetyltransferase and an increase of cholinesterase activity, suggesting that accumulation of Aβ disrupted cholinergic activity and caused the cognitive impairments of AD (Maurice *et al*., 1996; Ruan *et al*., 2009; Tohda *et al*., 2004). The deposition of Aβ in the pathogenesis of AD is invariably associated with oxidative stress and inflammatory responses. (Butterfield and Lauderback, 2002; Butterfield *et al*., 2007). Antioxidants such as α-tocopherol protect against Aβ-induced cytotoxicity in vitro as well as against development of learning and memory deficits *in vivo* (Yamada *et al*., 1999). Additionally, anti-inflammatory agents such as indomethacin reportedly slow the progression of AD (Gasparini *et al*., 2004). We hypothesized that CSF might protect neurons against neurodegeneration in AD due to its antioxidant and anti-inflammatory activities. The purpose of our study was to determine whether an ethanol extract of CSF have a protective effect against Aβ (25-35)-induced memory deficits in mice and Aβ (25-35)-induced neuronal damage in cultured rat cortical neurons.

MATERIALS AND METHODS

1. Plant materials and extraction

The dried and sliced CSF was purchased from Daegu Oriental Pharm Co. at Daegu, Korea and identified by one of the authors (Dr. Kyung-sik Song). A voucher specimen (KNUNPC-CSF-10-001) was deposited at Natural Products Chemistry Laboratory, Kyungpook National University, Daegu, Korea. CSF (2 ㎏) was refluxed with 20 L of 95% ethanol for 3 h and the extract was filtered through filter paper (Advantec MFS, CA, USA). The filtrate was concentrated to dryness under reduced pressure with a rotary evaporator to yield an ethanol extract (250 g), which was then stored at room temperature until required.

2. Experimental animals

Pregnant Sprague-Dawley (SD) rats for primary neuronal culture and male ICR mice for the passive avoidance test were supplied by Daehan BioLink Co., Ltd. (Chungbuk, Korea) and housed in an environmentally controlled room at 22 ± 2℃, with a relative humidity of 55 ± 5%, a 12-h light/dark cycle, and food and water *ad libitum*. The procedures involving experimental animals complied with the animal care guidelines of the National Institutes of Health and the animal ethics committee of Chungbuk National University.

3. Induction of neurotoxicity in primary cultures of rat cerebral cortical neurons

Primary cortical neuron cultures were prepared using embryonic day 15 to 16 SD rat fetuses, as previously described (Ban *et al*., 2005). Neurotoxicity experiments were performed on neurons after 3-4 days in culture. Cultured neurons were treated with 10 μM Aβ (25-35) (Bachem, Bubendorf, Switzerland) in serum-free Dulbecco's modified Eagle's medium (DMEM) (Sigma) at 37℃ for 36 h (unless otherwise indicated) to produce neurotoxicity. An Aβ (25-35) stock solution of 2 mM was prepared in sterile distilled water, stored at −20℃, and incubated for more than 2 days at 37℃ to aggregate before use. CSF was dissolved in dimethylsulfoxide (DMSO) at concentrations of 50 ㎎/㎖ and further diluted in experimental buffers. The final concentration of DMSO was ≤ 0.1%, which did not affect cell viability. For each experiment, CSF was applied 15 min prior to treatment with 10 μM Aβ (25-35). It was also present in the medium during Aβ (25-35) incubation.

4. Measurements of Aβ (25-35)-induced neuronal death and intracellular biochemical changes

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay and Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining were performed to measure neuronal death 36 h after exposure of cultured neurons to 10 μM Aβ (25-35), as described previously (Ban *et al*., 2005). Changes in $[Ca^{2+}]_i$ were measured with Fluo-4 AM (Molecular Probes), a calcium-sensitive fluorescent dye, using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) with a 488-㎚ excitation argon laser and 515-㎚ longpass emission filters (Ban *et al*., 2005). The microfluorescence of 2',7'-dichlorofluorescein, the fluorescent product of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes), and a laser scanning confocal microscope (MRC1024ES, Bio-Rad, Maylands, UK) with 488-㎚ excitation and 510-㎚ emission filters were used to monitor the generation of reactive oxygen species (ROS) in neurons treated with 10 μM Aβ (25-35) for 36 h (Ban *et al*., 2005).

5. Examination of learning and memory and measurement of brain cholinesterase activity

Induction of memory impairment in mice was performed by i.c.v. injection of the aggregated form of Aβ (25-35) (15 nmol), as previously described (Kim *et al*., 2009). CSF (10, 25, and 50 ㎎/㎏) suspended in distilled water was orally administered 30 min before the injection of Aβ (25-35) and further administered daily for 7 days. Memory acquisition was evaluated using step-through passive avoidance apparatus (Gemini Avoidance System, San Diego, CA, USA) according to the method previously described (Kim *et al*., 2009). At 30 min after administration of CSF on day 7 of i.c.v. injection of Aβ (25-35), mice were trained on step-through passive avoidance task. Retention trial was given 24 h after the training trial. After the retention trial of passive avoidance test, cholinesterase activity of mice whole brain was determined by the method of Hestrin (1949) with a slight modification as previously described (Cho *et al*., 2009).

6. Statistical analysis

Data are expressed as mean ± S.E.M.. Student's *t*-test was used for comparisons between two groups, and one-way analysis of variance (ANOVA) followed by Tukey's test was used for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

1. Effect of CSF on Aβ (25-35)-induced neuronal cell death

Based on our previous result (Lee *et al*., 2005), an Aβ (25-35) concentration of 10 μM was used for determining Aβ (25-35)-induced neuronal cell damage in the present study. When cortical neurons were exposed to 10 μM Aβ (25-35) for 36 h, absorbance in the MTT assay was 63.6 ± 2.6% of that of the untreated controls (Fig. 1), indicating that Aβ (25-35) caused neuronal cell death. In cultures treated with CSF (0.1, 1 and 10 ㎍/㎖), the Aβ (25-35)-induced neuronal death was significantly reduced (absorbance, 7.9 ± 1.9%, 81.1 ± 3.1%, and 85.2 ± 2.5% of control, respectively) (Fig. 1).

Hoechst 33342 staining was used to detect condensed or fragmented DNA, which is indicative of Aβ (25-35)-induced neuronal apoptotic death. Treatment of neurons with 10 μM Aβ (25-35) produced apoptosis of 32.7 ± 1.9% of the total population of cultured cortical neurons, as compared with 8.4 ± 1.2% of apoptotic neurons in control cultures. The addition of CSF (0.1, 1 and 10 ㎍/㎖) significantly decreased the Aβ (25-35)-induced apoptotic cell death, showing 23.6 ± 1.9%, 21.1 ± 1.6%, and 15.9 ± 1.8% of all neurons, respectively (Fig. 2).

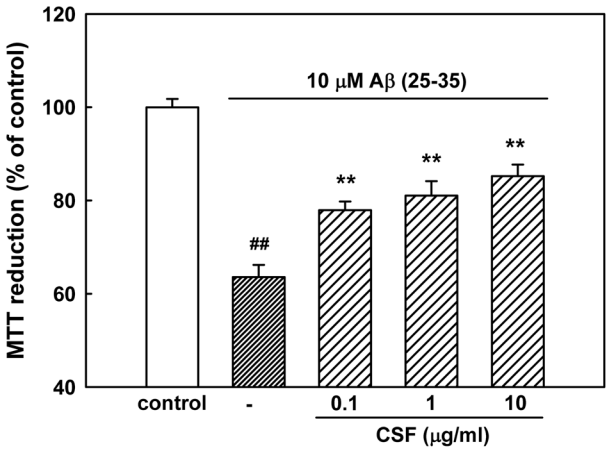

Fig. 1. Inhibitory effect of CSF on Aβ (25-35)-induced neuronal cell death in cultured cortical neurons. Neuronal cell death was measured using the MTT assay. The MTT absorbance from untreated cells was normalized to 100%. Results are expressed as mean ± S.E.M. of data obtained from 4 independent experiments. $^{\#\#}P < 0.01$ *vs* control (Student's *t*-test); $^{**}P < 0.01$ *vs* 10 μM Aβ (25-35) (Tukey's test).

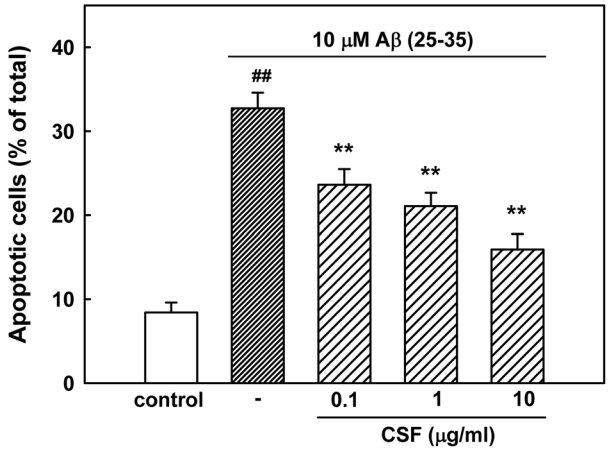

Fig. 2. Inhibitory effect of CSF on Aβ (25-35)-induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst 33342 staining were counted in 5 to 6 fields per well. The values represent the apoptotic cells as a percentage of the total number of cells expressed as mean ± S.E.M. of data obtained from 4 independent experiments. $^{\#\#}P < 0.01$ *vs* control (Student's *t*-test); $^{**}P < 0.01$ *vs* 10 μM Aβ (25-35) (Tukey's test).

2. Effect of CSF on Aβ (25-35)-induced $[Ca^{2+}]_i$ elevation

Aβ-induced cell death has been associated with an increases in $[Ca^{2+}]_i$. In our neuronal cultures, $[Ca^{2+}]_i$ showed an initial rapid

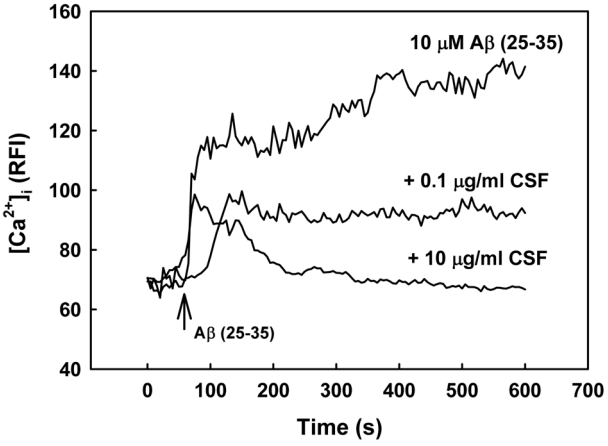

Fig. 3. Inhibitory effect of CSF on Aβ (25-35)-induced $[Ca^{2+}]_i$ elevation in cultured cortical neurons. $[Ca^{2+}]_i$ was monitored using Fluo-4 AM dye and a confocal laser scanning microscope. All images were processed to analyze changes in $[Ca^{2+}]_i$ at the single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace shows a single cell that is representative of at least 3 independent experiments.

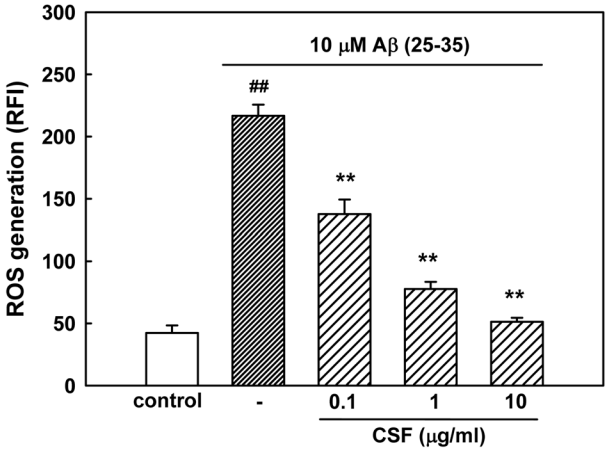

Fig. 4. Inhibitory effect of CSF on Aβ (25-35)-induced ROS generation in cultured cortical neurons. ROS was monitored using H_2DCF-DA dye and a confocal laser scanning microscope. Results are expressed as mean ± S.E.M. of RFI obtained from 3 independent experiments. $^{\#\#}P < 0.01$ *vs* control (Student's *t*-test); $^{**}P < 0.01$ *vs* 10 μM Aβ (25-35) (Tukey's test).

increase followed by a gradual increase in response to treatment with 10 μM Aβ (25-35) with intermittent fluctuations over 10 min (Fig. 3). In contrast, pretreatment with CSF (0.1 and 10 ㎍/㎖) showed a significant inhibition of the increase of $[Ca^{2+}]_i$ induced by 10 μM Aβ (25-35). CSF did not affect basal $[Ca^{2+}]_i$.

3. Effect of CSF on Aβ (25-35)-induced ROS generation

The involvement of oxidative stress in Aβ neurotoxicity was investigated by measurement of ROS accumulation after the exposure of the neurons to Aβ (25-35) for 36 h. In H_2DCF-DA-loaded cultured neurons, 10 μM Aβ (25-35) increased the fluorescence intensity, indicating that ROS were generated. In neurons treated with 10 μM Aβ (25-35), the relative fluorescence intensity increased approximately 5-fold to 216.7 ± 8.9 compared with the value in control neurons (42.4 ± 6.0). The Aβ (25-35)-induced increase of ROS generation was significantly inhibited by CSF (0.1, 1 and 10 ㎍/㎖) (Fig. 4).

4. Effect of CSF on Aβ (25-35)-induced memory impairment

In the initial acquisition trial of the passive avoidance task, the step-through latency did not differ among the four groups (data not shown). The step-through latency of the Aβ (25-35)-treated group in the retention trial significantly decreased to 97.4 ± 9.6 s,

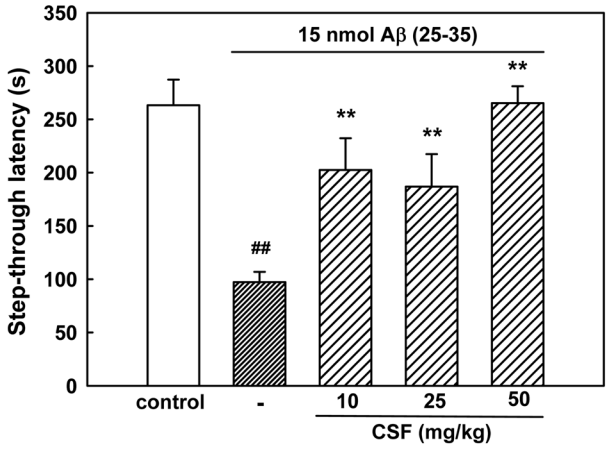

Fig. 5. Protective effect of CSF on Aβ (25-35)-induced memory impairment in mice. The learning and memory performance was assessed by the passive avoidance test. Values are expressed as mean ± S.E.M. of step-through latency (n = 8-12). $^{\#\#}P < 0.01$ *vs* sham-operated control (Student's *t*-test); $^{**}P < 0.01$ *vs* 15 nmol Aβ (25-35) (Tukey's test).

compared with 263.2 ± 24.1 s in the control group, indicating that Aβ (25-35) impaired memory in mice. Chronically administered CSF markedly protected against the memory impairment produced by Aβ (25-35). The step-through latency in groups administered CSF was 202.5 ± 29.8, 187.0 ± 30.4 and 265.3 ± 15.8 s at doses of 10, 25 and 50 ㎎/㎏, respectively (Fig. 5). We failed to find the dose-dependency of CSF.

Table 1. Effect of CSF on brain cholinesterase activity in mice.

Group	Dose	cholinesterase activity* (μmol/h g brain^{-1})
Control	–	672.7 ± 68.4
Aβ (25-35)	15 nmol/animal	710.5 ± 30.2
+ CSF	10 ㎎/㎏	652.6 ± 33.1
+ CSF	25 ㎎/㎏	616.9 ± 40.3
+ CSF	50 ㎎/㎏	545.5 ± 44.7*

*Results are expressed as mean ± S.E.M. of cholinesterase activity in brain (n = 6-8 mice/group). **P < 0.01 *vs* Aβ (25-35) alone (Tukey's test).

To determine the effect of CSF on cholinergic function, brain cholinesterase activity was measured. No significant increase of cholinesterase activity was shown in brains exposed to 15 nmol Aβ (25-35). However, chronic administration of 50 ㎎/㎏ CSF resulted in a significant reduction of cholinesterase activity showing 545.5 ± 44.7 mol/h/g brain compared with 710.5 ± 30.2 mol/h/g brain in Aβ (25-35)-treated brains (Table 1).

DISCUSSION

Aβ (25-35) forms a β-sheet structure and induces neuronal cell death, neuritic atrophy, synaptic loss, and memory impairment, as an active partial fragment of Aβ, although it is not found in the AD brain (Maurice *et al*., 1996; Tohda *et al*., 2004). Previously, we have reported that Aβ (25-35) caused neuronal cell death, which was blocked by treatment with (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), an NMDA antagonist, verapamil, a L-type Ca^{2+} channel blocker, and N^{G}-nitro-$_{L}$-arginine methyl ester ($_{L}$-NAME), a nitric oxide synthase (NOS) inhibitor (Ban *et al*., 2006; Lee *et al*., 2005). This result implies the involvement of NMDA-glutamate receptor activation, an increase of Ca^{2+} influx and generation of ROS in Aβ (25-35)-induced neurotoxicity in cultured cortical neurons, as evidenced in other studies (Ekinci *et al*., 2000; Gray and Patel, 1995; Ueda *et al*., 1997). The primary event following Aβ (25-35) treatment of cultured neurons has been suggested to be Ca^{2+} influx, apparently via L-type voltage-dependent Ca^{2+} channel (L-VDCC), since blockage of this channel and/or Ca^{2+} chelation prevents all other consequences (Ekinci *et al*., 2000; Ueda *et al*., 1997). It has been reported that vitamin-E, an antioxidant, blocked the Aβ-induced generation of ROS, but not Ca^{2+} influx, and reduction of extracellular Ca^{2+} inhibited the Aβ-induced increase in intracellular Ca^{2+} as well as generation of ROS, indicating that ROS generation is a consequence of Ca^{2+} accumulation (Ekinci *et al*., 2000). In the present study, Aβ (25-35) elicited gradual and significant $[Ca^{2+}]_i$ increase, ROS generation, and neuronal cell death in cultured cortical neurons, which were blocked by CSF. These results indicate that CSF might prevent Aβ (25-35)-induced Ca^{2+} entry through VDCC- and/or NMDA-receptor-coupled channels to inhibit ROS generation and then neuronal death. Many experiments, however, have demonstrated that free radicals are responsible for the increase of $[Ca^{2+}]_i$. The ROS-induced membrane damage causes further Ca^{2+} influx and resultantly accentuated Ca^{2+} influx in turn will induce the generation of further ROS (Cotman *et al*., 1992). CSF has been proven to be rich in antioxidant constituents such as flavonoids and vitamin C (Hamauz *et al*., 2005) Therefore, it also can be suggested that the CSF could inhibit the delayed $[Ca^{2+}]_i$ increase through suppression of ROS generation, and resultantly ameliorated Aβ (25-35)-induced neuronal death.

Many researchers have demonstrated that Aβ triggers apoptotic degeneration in *in vitro* neuronal experiments (Ekinci *et al*., 1999; Yan *et al*., 1999). In the present work, cultured cortical neurons exposed to Aβ (25-35) for 36 h showed increased chromatin condensation, a typical feature of apoptotic cell death, which was reduced by CSF. Activation of caspases after increased $[Ca^{2+}]_i$ signaling and ROS generation, or inflammatory responses in Aβ-stimulated neurons have been proposed to play pivotal roles in apoptosis (Costa *et al*., 2010; Gasparini *et al*., 2004). We have also demonstrated that an increase of caspase-3 activity in Aβ (25-35)-treated cultured cortical neurons is correlated with the increase of $[Ca^{2+}]_i$, ROS generation and neuronal apoptotic death (Ban *et al*., 2006; Lee *et al*., 2005). In the present study, CSF might inhibit caspase activity to reduce Aβ (25-35)-induced neuronal apoptosis. The molecular mechanism for the prevention of neuronal apoptosis by CSF should be further clarified.

I.c.v. injection of Aβ (25-35) into experimental rodents induces memory impairment in different behavioral paradigms, including spontaneous alternation, the water maze, and passive avoidance (Maurice *et al*., 1996; Um *et al*., 2006). Memory impairment in the passive avoidance test was also confirmed in mice 7 days after the i.c.v. injection of Aβ (25-35) in the present work. Chronic treatment with CSF effectively protected the mice against Aβ (25-35)-induced memory deficit. This result was consistent with its protective effect on Aβ (25-35)-induced neurotoxicity *in vitro*. Aβ accumulation associated with cognitive impairment in AD is accompanied by an increase in cholinesterase activity (Maurice *et al*., 1996). To slow the

progression of AD and improve cognitive function, it was proposed to restore the cholinergic balance through inhibition of acetylcholine breakdown by cholinesterase (Francis *et al*., 1999; van Marum, 2008). Cholinesterase inhibitors such as tacrine and donepezil have been developed for the treatment of cognitive loss in AD based on the cholinergic deficits hypothesis (van Marum, 2008). Although a significant increase of cholinesterase activity was not produced by Aβ (25-35), it was inhibited by CSF (50 ㎎/㎏) in Aβ (25-35)-treated mice brain in the present study. This result suggests that CSF may increase cholinergic activity and be able to treat memory impairment in AD.

On the other hand, elevated levels of Aβ induce oxidative stress, increasing the appearance of ROS such as superoxide and NO and subsequently producing $ONOO^-$ by a rapid interaction, could mediate the damage seen in AD (Kontush, 2001; Smith *et al*., 1997). A scavenger of $ONOO^-$ and antioxidants such as α-tocopherol protect against learning and memory deficits induced by Aβ (Alkam *et al*., 2007; Yamada *et al*., 1999). In the present study, 10 μM Aβ (25-35) significantly increased the ROS level in cultured neurons, and this was inhibited by CSF. In addition, CSF contains flavonoids as one of the main phytochemical constituents, which are effective in preventing free radical-caused oxidative damage (Zhou *et al*., 2007; Zhang *et al*., 2009). Therefore, it is possible that the favorable effect of CSF on Aβ (25-35)-induced cognitive deficits can be attributed to the inhibition of ROS generation. It has been reported that aqueous extract of CSF inhibited memory deficit induced by the intrahippocampal injection of Aβ (1-40) in mice through inhibition of cytokine expression and ROS generation (Jung and Lee, 2004). In their experiments, delayed memory impairment was produced through 10 weeks by injection of very low level of Aβ (1-40) (10 μM, 0.5 ㎕), and 284 ㎎/㎏ of CSF, which was more than 5 times higher than the highest dosage of 50 ㎎/㎏ used in the present study, was daily administered for 8 weeks. It was not until 4 weeks that the water extract of CSF revealed the inhibition of Aβ (1-40)-induced memory deficit in mice. We used an ethanol extract of CSF for 1 week to produce antidementia. These results may explain that there are considerable differences of active components between water and ethanol extract of CSF. The present results demonstrated a novel pharmacological activity, a protective effect on Aβ-induced neurotoxicity in cultured neurons, of an ethanol extract of CSF.

In conclusion, this study demonstrated that an ethanol extract of CSF protected against Aβ (25-35)-induced neuronal damage in cultured rat cortical neurons and memory impairment in mice. These results may explain the inhibitory action of CSF on the progression of AD. Further studies should determine the specific components of CSF that are responsible for preventing the neuronal damage.

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