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머루전초, 독활전초, 감초 혼합추출물의 Amyloid β Protein (25-35) 유발 신경독성에 대한 억제효과

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Inhibitory Effect of an Ethanol Extract Mixture of *Vitis amurensis, Aralia cordata,* and *Glycyrrhizae* radix on Amyloid β Protein (25-35)-Induced Neurotoxicity

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ABSTRACT : The present study investigated an ethanol extract (SSB) of a mixture of three medicinal plants of *Vitis amurensis, Aralia cordata,* and *Glycyrrhizae* radix for possible neuroprotective effects on neurotoxicity induced by Amyloid β protein (Aβ) (25-35) in cultured rat cortical neurons and antidementia activity in mice. Exposure of cultured cortical neurons to 15 μM Aβ (25-35) for 36 h induced neuronal apoptotic death. At 1-30 ㎍/㎖, SSB inhibited neuronal death, elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), and generation of reactive oxygen species (ROS) induced by Aβ (25-35) in cultured cortical neurons. Memory impairment and increase of acetylcholinesterase activity induced by intracerebroventricular injection of mice with 16 nmol Aβ (25-35) was inhibited by chronic treatment with SSB (25, 50 and 100 ㎎/㎏, p.o., for 8 days). From these results, it is suggested that antidementia effect of SSB is due to its neuroprotective effect against Aβ (25-35)-induced neurotoxicity and that SSB may have a therapeutic role in preventing the progression of Alzheimer's disease.

Key Words **:** *Vitis amurensis, Aralia cordata, Glycyrrhizae* radix, Amyloid β Protein, Cultured Neurons, Memory Impairment

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder, and the most common type of dementia. It is characterized by pronounced loss of neurons, extracellular senile plaque, whose principle component is β-amyloid (Aβ), a 39-43 amino acid peptide derived from the cleavage of the amyloid precursor protein by β and γ-secretase (Ivins *et al.*, 1999). Aβ can directly induce neuronal cell death (Ueda *et al.*, 1997) and cause neurons vulnerable to excitotoxicity (Koh *et al.*, 1990) and oxidative insults (Chen *et al.*, 2007). It is suggested that 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 resulting from reactive oxygen species (ROS) generation (Ekinci *et al.*, 2000; Gray and Patel, 1995; Ueda *et al.*, 1997). Aβ has been reported to produce hydrogen peroxide, superoxide, and proinflammatory cytokines in neurons and glial cells (Gitter *et al.*, 1995). Therefore, antioxidants such as α-tocopherol and anti-inflammatory agents such as indomethacin reportedly slow the progression of AD (Gasparini *et al.*, 2004; Sano *et al.*, 1997).

Vitis, known as a kind of grapes, has various pharmacological effects including improvement against fatty liver, hypertension, cardiac diseases and arthritis (Wang *et*

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al., 2004). *Vitis* contains large amount of polyphenolic compounds such as resveratrol possessing potent antioxidant properties (Rice-Evans *et al.*, 1995) and anti-inflammatory properties (Wang *et al.*, 2004). *Aralia* belongs to the families Araliaceae and has long been recognized as therapeutic herbs for antinociceptive, antidiabetic, antioxidant and anti-inflammatory activities in China, Japan and Korea (Bae, 2004). Diterpenes isolated from the root of this plant were known as active constituents for analgesic and anti-inflammatory agents (Han *et al.*, 1985). Recent studies have indicated that extracts from the aerial parts of *A. cordata* inhibit cyclo-oxygenase (COX)-1, COX-2, and COX-2-dependent prostaglandin E2 (PGE2) generation, as well as hyperalgesia during peripheral inflammation (Dang *et al.*, 2005; Lee *et al.*, 2006; Park *et al.*, 2005). *Glycyrrhizae* radix, the root of *Glycyrrhizae uralensis* (Lycophodiaceae), has been widely used in Chinese medicine and food. Current European and Chinese texts list *Glycyrrhizae* radix as an expectorant in the treatment of bronchitis, catarrh, and coughs, as an anti-inflammatory agent in gastric ulcers, arthritis, and rheumatism, and as an adrenocorticotropic agent in adrenocorticoid insufficiency (Huang, 1999). Moreover, *Glycyrrhizae* radix has been reported to have antioxidant effects (Tang *et al.*, 2004).

There has been much effort to develop beneficial agents from medicinal plants with free radical scavenging and anti-inflammatory properties to achieve neuroprotection in our laboratory (Jung *et al.*, 2012; Kim *et al.*, 2011). In our previous studies, aerial parts, leaves and stems, of *Vitis amurensis* and *Aralia cordata* were demonstrated to show neuroprotective effect against Aβ (25-35)-induced neurotoxicity in *in vivo* and *in vitro* (Cho *et al.*, 2009; Jeong *et al.*, 2010). *Glycyrrhizae* radix also showed neuroprotection in cultured neurons, but not in *in vivo* (Lee *et al.*, 2012). Therefore, we hypothetized that mixture of three plants might show more effective and synergistic neuroprotective effect with the reduction of possible individual toxicity due to large quantity.

The present study investigated the neuroprotective property of an ethanol extract of mixture of *Vitis amurensis, Aralia cordata,* and *Glycyrrhizae* radix, which was named as SSB, against Aβ (25-35)-induced memory deficits in mice and neuronal damage in cultured rat cortical neurons.

MATERIALS AND METHODS

1. Plant Materials and Extraction

The leaves and stems of *V. amurensis* and the aerial parts of *A. cordata* were collected at Keryong Mountain in Daejeon, Korea, from May to July and *Glycyrrhizae* radix (Korean) was purchased from Daegu Oriental Pharm Co. at Daegu, Korea. These plants were identified by Dr. KiHwan Bae, Chungnam National University. Each 200 g of the three plants was mixed, extracted with ethanol (3 L × 24 h × 3) at room temperature, filtered, and concentrated under reduced pressure using a rotary evaporator to yield an ethanol extract (SSB, 64 g) (Choung *et al.*, 2013), which was then stored at −20℃ 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 guide lines 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.*, 2006). Neurotoxicity experiments were performed on neurons after 3-4 days in culture. Cultured neurons were treated with 15 μM Aβ (25-35) (Bachem, Bubendorf, Switzerland) in serum-free DMEM (Sigma, St. Louis, MO, USA) 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. SSB was dissolved in dimethyl-sulfoxide (DMSO) at concentrations of 50 ㎎/㎖ and further diluted in experimental buffers. The final concentration of DMSO was less than 0.1%, which did not affect cell viability. For each experiment,

SSB was applied 20 min prior to treatment with 15 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, St. Louis, MO, USA) assay and Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining were performed to measure neuronal death 36 h after exposure of cultured neurons to 15 μM Aβ (25-35), as previously described (Ban *et al.*, 2006). Changes in $[Ca^{2+}]_i$ were measured with Fluo-4 AM (Molecular Probes), a Ca^{2+}-sensitive fluorescent dye, using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) with 488-nm excitation argon laser and 515-nm longpass emission filters. The microfluorescence of 2',7'-dichlorofluorescein, the fluorescent product of 2',7'-dichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes), and a laser scanning confocal microscope (MRC 1024ES, Biorad, Maylands, UK) with 488-nm excitation and 510-nm emission filters were used to monitor the generation of ROS in neurons treated with 15 μM Aβ (25-35) for 24 h.

5. Measurement of Memory Impairment and Brain Cholinesterase Activity

Intracerebroventricular (i.c.v.) injection of the aggregated Aβ (25-35) (16 nmol) was performed to induce memory impairment in mice, as previously described (Kim *et al.*, 2009). SSB (25, 50 and 100 ㎎/㎏) suspended in distilled water was orally administered to 5-week-old ICR mice 30 min before the injection of Aβ (25-35) and further administered daily for 7 days. Passive avoidance apparatus (Gemini Avoidance System, SanDiego, CA, USA) was used to measure memory acquisition according to the method previously described (Kim *et al.*, 2009). Mice were trained on step-through passive avoidance task 30 min after administration of SSB on day 7 of i.c.v. injection of Aβ (25-35). Retention trial was given 24 h after the acquisition trial. Cholinesterase activity of mice whole brain isolated immediately after the retention trial of passive avoidance test was determined using the spectrophotometric method (Ellman *et al.*, 1961).

6. Statistical Analysis

Data were expressed as mean ± SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's tests. P values of < 0.05 was considered significant.

RESULTS

1. SSB Inhibited Aβ (25-35)-Induced Neuronal Cell Death

Cultured cortical neurons exposed to 15 μM Aβ (25-35) for 36 h showed 60.5 ± 2.5% absorbance of that of the untreated controls in the MTT assay (Fig. 1), indicating that Aβ (25-35) caused neuronal cell death. Pretreatment of cortical neurons with 10 and 30 ㎍/㎖ SSB reduced the neuronal death induced by Aβ (25-35) (absorbance, 74.4 ± 2.8% and 82.0 ± 3.0% of control, respectively; Fig. 1).

An additional experiment was performed with Hoechst 33342 staining to detect condensed or fragmented DNA, which is indicative of Aβ (25-35)-induced neuronal apoptotic death. Treatment of neurons with 15 μM Aβ (25-35) produced apoptosis of 41.6 ± 2.7% of the total population of cultured cortical neurons, as compared with 13.4 ± 2.1% of apoptotic neurons in control cultures. The addition of SSB (1, 10 and 30 ㎍/㎖) significantly decreased the Aβ (25-35)-induced apoptotic cell death, showing 22.2 ± 3.2, 21.0 ± 2.1 and 16.2 ± 2.5%, respectively (Fig. 2).

2. SSB Inhibited Aβ (25-35)-Induced $[Ca^{2+}]_i$ Elevation

Increase in $[Ca^{2+}]_i$ has been associated with Aβ-induced cell death. In cultured neurons, $[Ca^{2+}]_i$ rapidly increased in response to treatment with 15 μM Aβ (25-35) and then showed a slow and gradual decrease over 10 min (Fig. 3). In contrast, pretreatment with SSB (30 ㎍/㎖) showed inhibition of the increase of $[Ca^{2+}]_i$ induced by 15 μM Aβ (25-35).

3. SSB inhibited Aβ (25-35)-Induced ROS Generation

To explain the involvement of oxidative stress in Aβ neurotoxicity, the accumulation of ROS after the exposure of the neurons to Aβ (25-35) for 24 h was measured. In H_2DCF-DA-loaded cortical neurons, 15 μM Aβ (25-35) increased the fluorescence intensity, indicating that ROS were generated. In neurons treated with 15 μM Aβ (25-

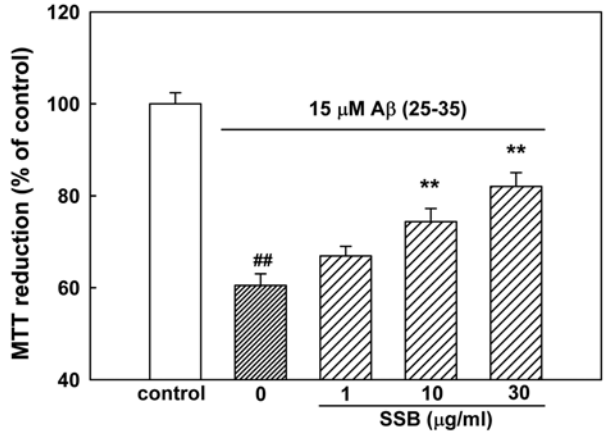

Fig. 1. Inhibitory effect of SSB on Aβ (25-35)-induced neuronal cell death in cultured cortical neurons. Neuronal cell death was measured using the MTT assay. The MTT absorbance from untreated cells was normalized to 100% as a control. Results are expressed as mean ± SEM of data obtained from 5 independent experiments. $^{\#\#}p<0.01$ *vs*. control; $^{**}p < 0.01$ *vs*. 15 μM Aβ (25-35).

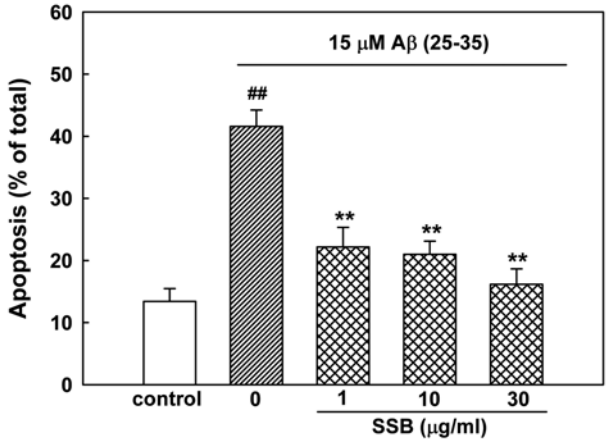

Fig. 2. Inhibitory effect of SSB on Aβ (25-35)-induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst 33342 staining were counted in 5 to 6 fields per well. The values represent the apoptotic cells as a percentage of the total number of cells expressed as mean ± SEM of data obtained from 3 independent experiments. $^{\#\#}p < 0.01$ *vs*. control; $^{**}p < 0.01$ *vs*. 15 μM Aβ (25-35).

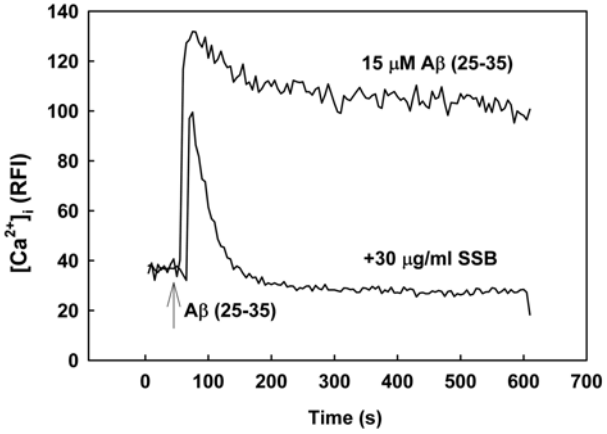

Fig. 3. Inhibitory effect of SSB on Aβ (25-35)-induced $[Ca^{2+}]_i$ elevation in cultured cortical neurons. $[Ca^{2+}]_i$ was monitored using Fluo-4 AM dye and a confocal laser scanning microscope. All images were processed to analyze changes in $[Ca^{2+}]_i$ at the single cell level. Results are expressed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

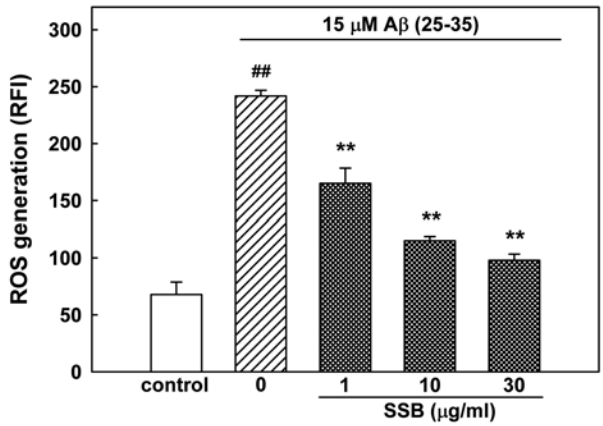

Fig. 4. Inhibitory effect of SSB on Aβ (25-35)-induced ROS generation in cultured cortical neurons. ROS was monitored using H_2DCF-DA dye and a confocal laser scanning microscope. Results are expressed as mean ± SEM of RFI obtained from 4 independent experiments. $^{\#\#}P < 0.01$ *vs*. control; $^{**}p < 0.01$ *vs*. 15 μM Aβ (25-35).

35), the relative fluorescence increased approximately 3.5-fold to 241.8 ± 5.0 compared with the value in control neurons (67.8 ± 10.9, Fig. 4). The Aβ (25-35)-induced increase in ROS generation was significantly inhibited by SSB (1, 10 and 30 ㎍/㎖).

4. SSB Inhibited 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 5 groups (control, 16 nmol Aβ (25-35), Aβ (25-35) + 25 ㎎/㎏ SSB, Aβ (25-35) + 50 ㎎/㎏ SSB, and Aβ (25-35) + 100 ㎎/㎏ SSB; data not shown). The step-through latency of the Aβ (25-35)-treated group in the retention trial significantly decreased to 31.6 ± 9.9 s, compared with 213.6 ± 30.3 s in the control group, indicating that Aβ (25-35) impaired memory in mice. Chronically administered SSB markedly protected against

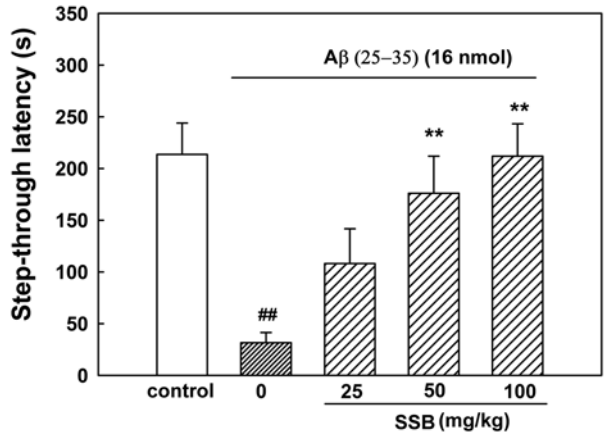

Fig. 5. Protective effect of SSB on Aβ (25-35)-induced memory impairment in mice. The learning and memory per-formance was assessed by the passive avoidance test. Values are expressed as mean ± SEM of step-through latency (n = 8-12). $^{\#\#}P < 0.01$ *vs*. sham-operated control; $^{**}p < 0.01$ *vs*. 16 nmol Aβ (25-35).

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

Group	Dose	cholinesterase activity (μmol/h mg protein^{-1})
Control	–	172.8 ± 7.1
Aβ (25-35)	16 nmol/animal	222.2 ± 9.9$^{\#\#}$
+ SSB	25 mg/kg	200.0 ± 9.2
+ SSB	50 mg/kg	181.1 ± 13.9*
+ SSB	100 mg/kg	163.7 ± 12.6**

Results are expressed as mean ± SEM of cholinesterase activity in brain (n = 8-12). $^{\#\#}p < 0.01$ *vs*. sham-operated control; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. 16 nmol Aβ (25-35).

the memory impairment produced by Aβ (25-35). The step-through latency in groups administered SSB was 108.1 ± 33.6, 176.0 ± 35.9 and 212.0 ± 31.2 s at doses of 25, 50 and 100 mg/kg, respectively (Fig. 5).

Cholinesterase activity in brain exposed to 16 nmol Aβ (25-35) was significantly increased. In the group consuming 50 mg/kg and 100 mg/kg SSB, the cholinesterase activity significantly decreased compared with the Aβ (25-35) group (Table 1).

DISCUSSION

Aβ (25-35), which is the core toxic fragment of full length Aβ (1-40), forms a β-sheet structure and induces neuronal cell death, neuritic atrophy, synaptic loss, and memory impairment (Maurice *et al.*, 1996; Pike *et al.*, 1995; Tohda *et al.*, 2004). It has been reported that Aβ (25-35) causes neuronal cell death as shown in the present study, and it was blocked by MK-801, an NMDA receptor antagonist; verapamil, a L-type Ca^{2+} channel blocker; and and N^{G}-nitro-$_{L}$-arginine methyl ester ($_{L}$-NAME), a nitric oxide synthase inhibitor, in cultured neurons (Cho *et al.*, 2009; Jeong *et al.*, 2010). These results suggest that NMDA glutamate receptor activation, Ca^{2+} influx through L-type voltage dependent Ca^{2+} channel (L-VDCC) and ROS generation are implicated in Aβ-induced neuronal apoptotic death. The present study provides evidence that Aβ (25-35)-induced injury to rat cortical neurons was prevented by SSB, an ethanol extract of a mixture of *Vitis amurensis, Aralia cordata,* and *Glycyrrhizae* radix. SSB was able to reduce the $[Ca^{2+}]_i$ increase, ROS generation, and apoptotic neuronal death in Aβ (25-35)-induced neurotoxicity. Additionally, SSB prevented memory loss induced by i.c.v. injection of Aβ (25-35) in mice.

Ca^{2+} influx via L-VDCC is considered as a primary insult after Aβ treatment in cultured neurons, because blockade of this channel or Ca^{2+} chelation prevents other consequences (Ueda *et al.*, 1997). In the present study, SSB inhibited a significant increase of $[Ca^{2+}]_i$ after the treatment with Aβ (25-35). Many reports have demonstrated the role of ROS formation in Aβ-induced neurotoxicity (Miranda *et al.*, 2000; Morais Cardoso *et al.*, 2002). 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 $[Ca^{2+}]_i$ as well as generation of ROS, indicating that ROS generation is a consequence of Ca^{2+} accumulation (Ekinci *et al.*, 2000). SSB also decreased the Aβ (25-35)-induced increase of ROS generation. These results indicate that SSB 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, although the mechanism by which SSB blocks the channels is not clear. *Vitis amurensis, Aralia cordata,* and *Glycyrrhizae* radix, the constituents of SSB, have been reported to possess antioxidant principles, resveratrol, oleanolic acid, and isoliquirtigenin (Du and Ko, 2006; Ha *et al.*, 2009a, b; Kumar *et al.*, 2007), respectively, suggesting that inhibition of Aβ (25-35)-induced neuronal death by SSB might be due to their ROS scavenging activity. Further

study should be conducted to elucidate the precise mechanism.

Many studies have demonstrated that Aβ is accompanied by multiple events culminating in apoptosis in *in vitro* neuronal experiments (Ekinci *et al.*, 2000; 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 SSB. 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. We have already 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 neural apoptotic death (Ban *et al.*, 2006; Lee *et al.*, 2005). In the previous studies, *Vitis amurensis, Aralia cordata,* and *Glycyrrhizae* radix inhibited caspase activity to reduce Aβ (25-35)-induced neuronal apoptosis (Cho *et al.*, 2009; Jeong *et al.*, 2010; Lee *et al.*, 2012). Therefore, SSB might work in the same way.

I.c.v. administration of Aβ (25-35) into mouse brain induces memory impairment in different behavioral paradigms, including spontaneous alternation, the water maze, and passive avoidance (Maurice *et al.*, 1996; Um *et al.*, 2006). Aβ (25-35) preferentially impairs spatial and non-spatial short-term memory, and these effects remain evident up to 6 months after even a single i.c.v. injection of the peptide (Stepanichev *et al.*, 2003). In the present work, memory impairment in the passive avoidance test was also confirmed in mice 7 days after the i.c.v. injection of Aβ (25-35). Chronic treatment with SSB effectively protected the mice against Aβ (25-35)-induced memory deficit. This result was essentially in agreement 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 (Atack *et al.*, 1983). Equivalently, the increase of cholinesterase activity by Aβ (25-35) in the brain was inhibited by SSB in the present study.

Many reports have suggested that oxidative stress is responsible for the onset of the cognitive dysfunction as well as the progression of AD (Butterfield *et al.*, 2001; Kontush, 2001). 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 $ONOO^-$ protects against Aβ (25-35)-induced memory impairment (Alkam *et al.*, 2007), and anti-oxidants such as α-tocopherol protect against cytotoxicity in vitro as well as against learning and memory deficits induced by Aβ (Sano *et al.*, 1997). In the present study, 15 μM Aβ (25-35) significantly increased the ROS level in cultured neurons, and this was inhibited by SSB. In addition, the constituents of SSB contain antioxidant components (Du and Ko, 2006; Ha *et al.*, 2009a,b; Kumar *et al.*, 2007). Therefore, it is possible that the favorable effect of SSB on Aβ (25-35)-induced cognitive deficits can be attributed to the inhibition of ROS generation.

In the previous studies (Cho *et al.*, 2009; Jeong *et al.*, 2010; Lee *et al.*, 2012), *Vitis amurensis, Aralia cordata*, and *Glycyrrhizae* radix were demonstrated to show neuroprotective effect against Aβ (25-35)-induced neurotoxicity in cultured neurons. Furthermore, *Vitis amurensis* (Jeong *et al.*, 2010) and *Aralia cordata* (Cho *et al.*, 2009) but not *Glycyrrhizae* radix, at the dosage of 50 and 100 ㎎/㎏ revealed protection against Aβ (25-35)-induced memory impairment in mice. Mixture of three plants, SSB, showed a maximal inhibition of Aβ (25-35)-induced memory impairment by a dosage of 100 ㎎/㎏ in the present study. These results indicate that three medicinal plants acted synergistically to produce neuroprotection, since maximal effect of SSB was shown at much lower concentration than the sum of maximal effect concentration of each plant.

In conclusion, it is evident that SSB could provide a marked protection against Aβ (25-35)-induced neuronal cell damage *in vitro* and Aâ (25-35)-induced memory deficit *in vivo*. These may explain the inhibitory action of SSB on the progression of AD.

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