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# Protective Effect of Sanguisorba officinalis L. Root on Amyloid β Protein (25-35)-induced Neuronal Cell Damage in Cultured Rat Cortical Neuron

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**ABSTRACT : Sanguisorbae radix (SR) from Sanguisorba officinalis L. (Losaceae) is widely used in Korea and China due to its various pharmacological activity. The present study aims to investigate the effect of the methanol extract of SR on amyloid β protein (25-35) (Aβ (25-35)), a synthetic 25-35 amyloid peptide, -induced neurotoxicity using cultured rat cortical neurons. SR, over a concentration range of 10-50 ㎍/㎖, inhibited the Aβ (25-35) (10 μM)-induced neuronal cell death, as assessed by a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and the number of apoptotic nuclei, evidenced by Hoechst 33342 staining. Pretreatment of SR (50 ㎍/㎖) inhibited 10 μM Aβ (25-35)-induced elevation of cytosolic calcium concentration ($[Ca^{2+}]_C$), which was measured by a fluorescent dye, fluo-4 AM. SR (10 and 50 ㎍/㎖) inhibited glutamate release into medium induced by 10 μM Aβ (25-35), which was measured by HPLC, and generation of reactive oxygen species. These results suggest that SR prevents Aβ (25-35)-induced neuronal cell damage *in vitro*.**

***Key words*** : Sanguisorbae radix, Neuroprotection, amyloid β protein (25-35); Neurotoxicity; Cortical neurons

## INTRODUCTION

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* (Iversen *et al.*, 1995) and in vivo (Chen *et al.*, 1994) studies have reported the toxic effects of Aβ or Aβ peptide fragments suggesting an important role of Aβ in the pathogenesis of AD. In cultures, Aβ can directly induce neuronal cell death (Ueda *et al.*, 1994) and can render neurons vulnerable to excitotoxicity (Koh *et al.*, 1990) and oxidative insults (Goodman & Matton, 1994). 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 (Forloni, 1993; Gray & Patel, 1995; Ueda *et al.*, 1997; Ekinci *et al.*, 2000). NMDA receptor acts either as a selective substrate of Aβ binding or as a mediator of Aβ-triggered glutamate excitotocixity (Harkany *et al.*, 1999). NMDA receptor is a ligand-gated/voltage-sensitive cation channel, especially highly permeable to $Ca^{2+}$. Extensive elevation of the $[Ca^{2+}]_i$ may lead directly to cellular dysfunction, overexcitation or death (Horn *et al.*, 1999). Therefore, $Ca^{2+}$ influx through NMDA receptor activation by Aβ exposure may be a critical role in Aβ-induced neurotoxicity, as proved by a report that the neurotoxic effect of Aβ was reduced by NMDA receptor antagonist, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine (MK-801) (Tibor *et al.*, 1999). Several lines of evidence support the involvement of oxidative stress as an active factor in Aβ-mediated neuropathology, by triggering or facilitating neurodegeneration through a wide range of molecular events that disturb neuronal homeostasis (Ekinci *et al.*, 2000). However, the clinical benefit of NMDA receptor antagonists and direct blockers of neuronal $Ca^{2+}$ channels is debatable, since they lack convincing effectiveness or have serious side-effects (Ferger & Krieglstein, 1996; Li *et al.*, 2002). There are much efforts to develop beneficial agents from medicinal plants to achieve neuroprotection.

Sanguisorbae radix (SR) is the dried root of Sanguisorba officinalis L. (Rosaceae), has hemostatic, analgesic, and astringent properties, and has been used in traditional oriental medicine for the treatment of burns, scalds, and internal hemorrhage (Cheong & Cao, 1992). It has been also reported to have anti-allergic activity (Shin *et al.*, 2002). However, there is not

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enough experimental evidence to prove the medicinal effect on the central nervous system (CNS) of SR. In a recent report, it was demonstrated that SR and its effective components inhibited the activity of β-secretase1, which is known as the most attractive target for the inhibition of Aβ production in AD (Lee *et al*, 2005b). In the course of screening natural products having anti-dementia activity, SR showed a significant preventive effect against the Aβ-induced neurotoxicity in cultured neuron. Therefore, to extend the knowledge on the pharmacological actions of SR in the CNS, the present study examined the neuroprotective effect of methanol extract of SR against Aβ (25-35)-induced neuronal death and the underlying mechanism in primarily cultured rat cortical neurons.

## MATERIALS AND METHODS

### Materials

SR was purchased from an oriental drug store in Taegu, Korea, and identified by professor K.-S. Song, Kyungpook National University. Aβ (25-35) was purchased from Bachem (Bubendorf, Switzerland). 2-Mercaptoethanol, trypsin (from bovine pancreas), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), o-phthaldialdehyde (OPA), Dulbecco's modified Eagle's medium (DMEM), Joklik-modified Eagle's medium, poly-L-lysine and amino acids for HPLC standard were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-4 AM, 2',7'-dichlorodihydrofluorescin diacetate ($H_2$DCF-DA) and Hoechst 33342 dye were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from JRH Biosciences (Lenexa, KS, USA). All other chemicals used were of the highest grade available.

### Preparation of methanol extract of SR

SR (300 g) was extracted three times in a reflux condenser for 24 h each with 2 ℓ of methanol. The solution was combined, filtered through Whatman NO. 1 filter paper, concentrated using a rotary vacuum evaporator, and freeze-dried. The yield was about 10% (w/w).

### Primary culture of cortical neurons

Primary cortical neuronal cultures were prepared using SD rat fetuses on embryonic day 16 ± 1. Fetuses were isolated from a dam anaesthetized with ether. Cortical hemispheres were dissected under sterile conditions and placed into Joklik-modified Eagle's medium containing trypsin (0.25 ㎎/㎖). After slight trituration through a 5-㎖ pipette 5 to 6 times, the cells were incubated for 10 min at 37℃ Dissociated cells were collected by centrifugation (1,500 rpm, 5 min) and resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), L-glutamine (3.64 mM), sodium bicarbonate (44 mM), glucose (22.73 mM), penicillin (40 U/㎖), gentamicin (50 ㎍/㎖), KCl (5 mM) and 10% fetal bovine serum at a density of about $2\times10^6$ cells/㎖. Cells were plated onto poly-L-lysine coated 12 well-plates (Corning 3512, NY, USA) for the measurements of cell death and glutamate release, and coverslips (Fisher Scientific 12CIR, Pittsburgh, PA, USA) for the measurements of cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$), reactive oxygen species (ROS) and apoptosis. After 2 days' incubation, the medium was replaced with a new growth medium in which the concentrations of fetal bovine serum and KCl were changed to 5% and 15 mM, respectively. Cultures were kept at 37℃ in a 5% $CO_2$ atmosphere, changing the medium twice a week. Immunochemical staining of cultures, when grown for 5-7 days *in vitro*, with anti-microtubule associated protein-2 (MAP-2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody revealed that the culture method used in this study provided cell cultures containing about 90% neurons.

### Neurotoxicity experiments

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. SR was dissolved in methanol with the concentration of 50 ㎎/㎖ and further diluted with experimental buffers. The final concentration of methanol was 0.1%, which did not affect cell viability. Neurotoxicity experiments were performed on neurons grown for 5-7 days *in vitro*. The culture medium was removed and replaced with serum-free growth medium. Cells were then incubated for 20 min in the medium, and incubated for a further 24 h (unless otherwise indicated) in the presence of 10 μM Aβ (25-35) at 37℃ to produce neurotoxicity. SR was applied to cells 15 min before the treatment with Aβ (25-35), and was also present in the medium during the incubation period with Aβ (25-35). For some experiments, a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl and 2.3 mM $CaCl_2$ at pH 7.4 was used.

### Analysis of cell viability

#### MTT colorimetric assay

This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductase (Lee *et al*., 2005a). Therefore, the amount of formazan produced is proportional to the number of viable cells. After completion of incubation with 10 μM Aβ (25-35) for 24 h, the culture medium was replaced by a solution of MTT (0.5 ㎎/㎖) in serum-free growth medium. After a 4 h incubation at 37℃, this solution was removed, and the resulting blue formazan was solubilized in 0.4 ml of acid-isopro-

panol (0.04 N HCl in isopropanol), and the optical density was read at 570 ㎚ using microplate reader (Bio-Tek ELX808, VT, USA). Serum-free growth medium was used as a blank solution.

**Measurement of apoptotic cell death**

The bis-benzimidazole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in cells without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy (Otto & Tsou, 1985). Exposed to 10 μM Aβ (25-35) in serum-free growth medium for 24 h, neurons on coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min, then stained with Hoechst 33342 dye at the concentration of 1 ㎍/㎖ in the incubation buffer for 15 min. The morphological change was examined under UV illumination using a fluorescence microscope (Olympus IX70-FL, Tokyo, Japan). The dye was excited at 340 ㎚, and emission was filtered with a 510 ㎚ barrier filter. To quantify the apoptotic process, neurons with fragmented or condensed DNA and normal DNA were counted. Data were shown as apoptotic cells as a percentage of total neurons.

### Measurement of $[Ca^{2+}]_c$

Neurons grown on coverslips were loaded with 3 μM fluo-4 AM (dissolved in dimethylsulfoxide (DMSO)) in serum-free growth medium for 45 min at 37 ℃ in the $CO_2$ incubator, and washed with the incubation buffer. The coverslips containing fluo-4 AM labeled cells were mounted on a perfusion chamber containing incubation buffer, subjected to a laser scanning confocal microscope (Carl Zeiss LSM 510, Oberkochen, Germany), and then scanned every 3 second with a 488 ㎚ excitation argon laser and a 515 ㎚ longpass emission filter. After the baseline of $[Ca^{2+}]_c$ was observed for 50 sec, 10 μM Aβ (25-35) was added to the perfusion chamber for the measurement of $[Ca^{2+}]_c$ change. In order to test the effect of SR on the Aβ (25-35)-induced $[Ca^{2+}]_c$ change, neurons were pretreated with SR (50 ㎍/㎖) 15 min before the treatment with 10 μM Aβ (25-35) after being loaded with fluo-4 AM and washed. SR was also present in the perfusion chamber during the $[Ca^{2+}]_c$ measurement period. All images, about 100 images from the scanning, were processed to analyze changes of $[Ca^{2+}]_c$ in a single cell level. The results were expressed as the relative fluorescence intensity (RFI) (Lee *et al.*, 1998).

### Measurement of glutamate concentration

After being washed and equilibrated for 20 min with the incubation buffer, neurons were incubated with the buffer containing 10 μM Aβ (25-35) for 6 h at 37 ℃ At the end of the incubation, glutamate secreted into the medium from the treated cells was quantified by high performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (BAS MF series, IN, USA) (Ellison *et al.*, 1987). Briefly, after a small aliquot was collected from the culture wells, glutamate was separated on an analytical column (ODS2; particle size, 5 ㎛; 4.6×100 ㎜) after pre-derivatization with OPA/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ㎖/min.

### Measurement of ROS generation

The microfluorescence assay of 2',7'-dichlorofluorescin (DCF), the fluorescent product of $H_2$DCF-DA, was used to monitor the generation of ROS. Neurons grown on coverslips were washed with phenol red-free DMEM 3 times and incubated with the buffer containing 10 μM Aβ (25-35) at 37 ℃ for 24 h. The uptake of $H_2$DCF-DA (final concentration, 5 μM) dissolved in DMSO was carried out for the last 10 min of the incubation with 10 μM Aβ (25-35). After being washed, coverslips containing cortical cells loaded with $H_2$DCF-DA were mounted on the confocal microscope stage, and the neurons were observed by a laser scanning confocal microscope (Bio-Rad, MRC1024ES, Maylands, UK) using 488 ㎚ excitation and 510 ㎚ emission filters. The average pixel intensity of fluorescence was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer. Challenge of $H_2$DCF-DA and measurement of fluorescence intensity was performed in the dark.

### Statistical analysis

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

## RESULTS

### SR protects neurons against cell death induced by Aβ, (25-35).

To assess Aβ (25-35)-induced neuronal cell death, the MTT assay was performed. MTT assay is extensively used as a sensitive, quantitative and reliable colorimetric assay for cell viability. In previous experiments (Ban & Seong, 2005), we have

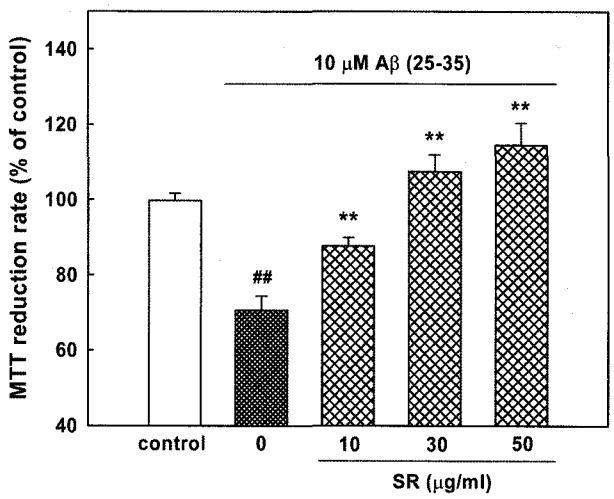


**Fig. 1.** Inhibitory effect of SR on Aβ (25-35)-induced cell death in cultured cortical neurons. Neuronal death was measured by the MTT assay. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean± SEM values of the data obtained from three independent experiments performed in triplicate. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 μM Aβ (25-35).

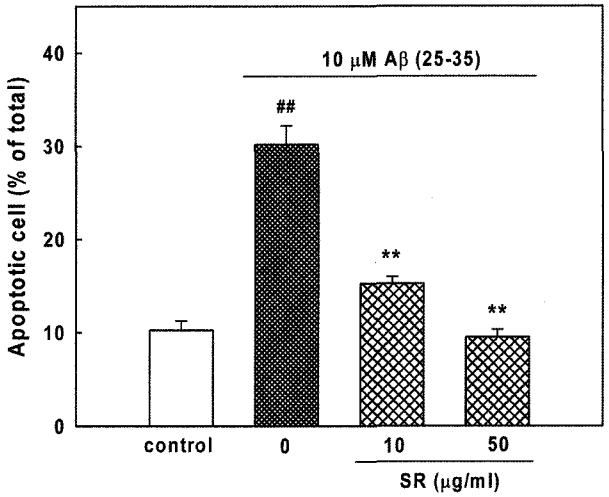


**Fig. 2.** Inhibitory effect of SR on Aβ (25-35)-induced apoptosis of cultured cortical neurons as measured by Hoechst 33342 staining. Apoptotic cells produced by 10 μM Aβ, (25-35) were counted from five to six fields per well. Results are shown as apoptotic cells as a percentage of total number of cells and expressed as mean±SEM values of the data obtained from four independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 μM Aβ (25-35).

demonstrated that Aβ (25-35) over the concentration range of 5-20 μM produced a concentration-dependent reduction of cell viability in cultured cortical neuron. Therefore, the concentration of 10 μM was used for the determination of Aβ (25-35)-induced neuronal cell damage in the present experiments. Fig. 1 shows the inhibitory effect of SR on a 10 μM Aβ (25-35)-induced decrease of MTT reduction. MTT reduction rate decreased to 70.6 ± 3.8% when using 10 μM Aβ (25-35). SR concentration-dependently reduced the Aβ (25-35)-induced decrease of MTT reduction over a concentration range of 10-50 ㎍/㎖ showing 114.5 ± 5.9% with 50 ㎍/㎖, a complete protection against the neuronal damage.

An additional experiment was performed with Hoechst 33342 staining to assess the neurotoxicity of Aβ (25-35). Cell nuclei stained by Hoeschst 33342 enables the occurrence of DNA condensate to be detected, a feature of apoptosis. In neurons treated with 10 μM Aβ (25-35), chromatin condensation and nuclear fragmentation were observed, whereas the control culture had round blue nuclei of viable cells. As shown in Fig. 2, the proportion of apoptotic cells was calculated. The treatment of cells with 10 μM Aβ (25-35) produced apoptosis of 30.2 ± 2.0% of the total population of cultured cortical neurons, as compared with 10.3 ± 1.0% of apoptotic neurons in control cultures. SR (50 ㎍/㎖) significantly decreased the Aβ (25-35)-induced apoptotic cell death, showing 9.5 ± 0.8%. SR alone did not affect cell viability (data not shown).

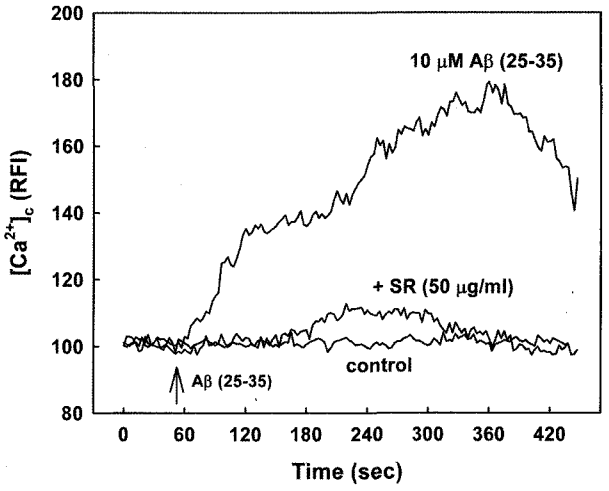


**Fig. 3.** Change of $[Ca^{2+}]_c$ in response to Aβ (25-35) in the presence or absence of SR in cultured cortical neurons. $[Ca^{2+}]_c$ was monitored using a laser scanning confocal microscope. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative.

### SR inhibits Aβ (25-35)-induced elevation of $[Ca^{2+}]_c$

The increase of $[Ca^{2+}]_c$ has been postulated to be associated with Aβ-induced cell death in many studies. In cultured cortical neurons, treatment with 10 μM Aβ (25-35) produced relatively slow and gradual increase of $[Ca^{2+}]_c$. A maximal fluores-

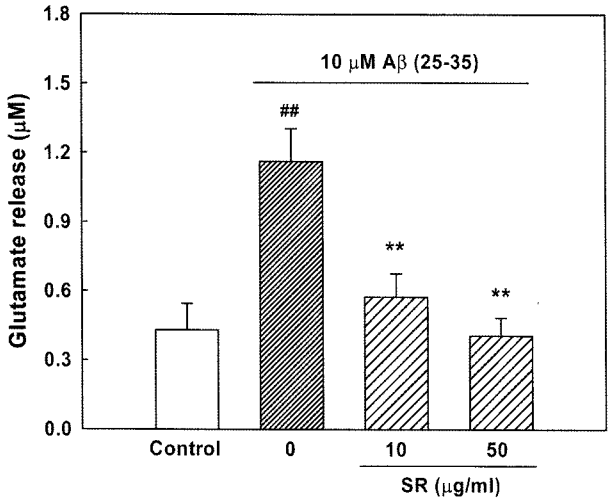


**Fig. 4.** Inhibitory effect of SR on Aβ (25-35)-induced glutamate release in cultured cortical neurons. The amount of released glutamate was measured by HPLC with ECD. Results are expressed as mean±SEM values of the data obtained in three independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 μM Aβ (25-35).

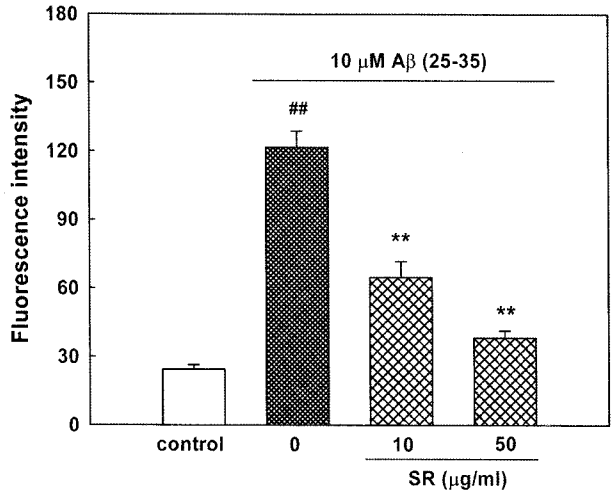


**Fig. 5.** Inhibitory effect of SR on Aβ (25-35)-induced ROS generation in cultured cortical neurons. Results are expressed as mean±SEM of relative fluorescence intensity obtained from three independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 μM Aβ (25-35).

cence intensity of about 180, compared to a base of 100, with the $[Ca^{2+}]_c$ elevation was measured about 5 min after the Aβ (25-35) application. After peaking, the fluorescence level was decreased gradually. In contrast, pretreatment with SR (50 ㎍/㎖) significantly inhibited the elevation of $[Ca^{2+}]_c$ induced by 10 μM Aβ (25-35) throughout the measurement period (Fig. 3). SR did not affect basal $[Ca^{2+}]_c$ (data not shown).

### SR inhibits Aβ (25-35)-induced elevation of glutamate release

Glutamate released into the extracellular medium was quantified 6 h after the incubation of cells with 10 μM Aβ (25-35). As shown in Fig. 4, 10 μM Aβ (25-35) markedly elevated the basal glutamate (r.t. 7.5 min) level from 0.43 ± 0.12 μM of control cells to 1.16 ± 0.14 mM. SR (50 ㎍/㎖) strongly blocked the Aβ (25-35)-induced elevation of glutamate release showing 0.41 ± 0.08 μM.

### SR inhibits Aβ (25-35)-induced ROS generation

Aβ (25-35) increased the glutamate release and the concentration of $[Ca^{2+}]_c$. Furthermore, the pathological condition induced by Aβ (25-35) is associated with accelerated formation of ROS. In Aβ (25-35) (10 μM)-treated cells for 24 h, the fluorescence intensity increased about 5 folds to 121.7 ± 7.1 compared to control cells of 24.5 ± 1.9. The Aβ (25-35)-induced increase of ROS generation was significantly inhibited by SR (50 ㎍/㎖) showing 38.3. ± 3.1 of fluorescence intensity (Fig. 5). SR alone did not show direct reaction with $H_2$DCF-DA to generate fluorescence (data not shown).

## DISCUSSION

Aβ is a major contributor to the pathogenesis of AD. Aβ-induced neurotoxicity has been attributed in various studies to $Ca^{2+}$ influx, and generation of ROS (Behl *et al.*, 1994; Arias *et al.*, 1995; Miranda *et al.*, 2000). Our previous studies confirmed that Aβ (25-35) caused neuronal cell death, which was blocked by the treatment with MK-801, verapamil, an L-type $Ca^{2+}$ channel blocker, and $N^G$-nitro-$_L$-arginine methyl ester ($_L$-NAME), a nitric oxide synthase (NOS) inhibitor (Ban & Seong, 2005; Lee *et al.*, 2005a). 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 (Gray & Patel, 1995; Ueda *et al.*, 1997; Ekinci *et al.*, 2000). Regardless of the relative contribution of these events to Aβ (25-35)-induced neurotoxicity, the primary event following Aβ (25-35) treatment in 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.*, 1999; Ueda *et al.*, 1997). Furthermore, Aβ (25-35)-induced elevation of $[Ca^{2+}]_c$ and neurotoxicity were inhibited by MK-801, suggesting $Ca^{2+}$ influx through NMDA receptor-coupled VDCC plays a critical role in the neurotoxicity (Tibor *et al.*, 1999). 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). Confirming these reports, it was demonstrated that the significant increase of ROS generation took more than 1 h, while the elevation of $[Ca^{2+}]_c$ occurred within seconds after the treatment with 10 μM Aβ (25-35). In addition, $_L$-NAME, a NOS inhibitor, failed to inhibit the Aβ (25-35)-induced increase in $[Ca^{2+}]_c$ in the short period of measurement in contrast to the complete inhibition of verapamil on the Aβ (25-35)-induced ROS generation in the previous data (Ban & Seong, 2005). However, in many experiments, free radicals are responsible for the increase of $[Ca^{2+}]_i$. The ROS-induced membrane damage causes further $Ca^{2+}$ influx and resultant accentuated $Ca^{2+}$ influx in turn will induce the generation of further ROS (Cotman *et al.*, 1992). Many researchers have demonstrated that Aβ, triggered apoptotic degeneration in *in vitro* neuronal experiment (Harkany *et al.*, 1999; Yan *et al.*, 1999). Cultured cortical neurons exposed to 10 μM Aβ (25-35) for more than 24 h showed increased chromatin condensation, a typical feature of apoptotic cell death in the present work. The Aβ (25-35)-induced apoptotic neuronal death was also blocked by MK-801, verapamil and $_L$-NAME (Lee *et al.*, 2005a; Ban & Seong, 2005).

The present study provides evidence that Aβ (25-35)-induced injury to rat cortical neurons can be prevented by SR. SR was able to reduce the Aβ (25-35)-induced neuronal apoptotic death, $[Ca^{2+}]_c$ increase, glutamate release, and ROS generation. Many reports demonstrated that Aβ neurotoxicity is mainly due to massive $Ca^{2+}$ influx through VDCC probably coupled to NMDA receptor (Ueda *et al.*, 1997; Harkany *et al.*, 1999). In the present study, Aβ (25-35) elicited gradual and significant $[Ca^{2+}]_c$ increase, which was blocked by SR. SR also significantly inhibited the Aβ (25-35)-induced glutamate elevation. This result indicates that the sustained inhibition on $[Ca^{2+}]_c$ elevation by SR resulted in the decrease of the Aβ, (25-35)-induced glutamate release. The elucidation of the variety of events occurring downstream of neuronal $Ca^{2+}$ overloading is still a matter for further research. ROS generation undoubtedly takes place in glutamate neurotoxicity and is likely due to $Ca^{2+}$ influx in the cytosol (Pereira & Oliveira, 2000). Many reports demonstrated the involvement of ROS formation in Aβ-induced neurotoxicity (Miranda *et al.*, 2000; Cardoso *et al.*, 2002). SR decreased the Aβ, (25-35)-induced increase of ROS generation. It was not elucidated whether SR suppressed ROS generation through the inhibition of $[Ca^{2+}]_c$ increase, or vice versa, in the present study. We demonstrated that $_L$-NAME, an inhibitor of ROS generation, failed to show an inhibition on the Aβ (25-35)-induced $[Ca^{2+}]_c$ increase occurred in seconds to minutes, while verapamil, a $Ca^{2+}$ channel antagonist, completely blocked ROS generation in a previous experiment using cultured cortical neurons (Ban & Seong, 2005). Therefore, it is suggested that SR inhibited the Aβ (25-35)-induced ROS generation via the blockade of $[Ca^{2+}]_c$ increase. It is thus concluded that SR may prevent the Aβ (25-35)-induced apoptosis of neuronal cell by interfering with the increase of $[Ca^{2+}]_c$, and then by inhibiting glutamate release and generation of ROS.

Aβ is believed to play a central role in the pathophysiology of AD (Hsiao *et al.*, 1995; Holcomb *et al.*, 1998). Although it is still controversial whether increased Aβ formation is sufficient to cause nerve cell degeneration in AD, neurotoxic effects of Aβ have been demonstrated in both *in vitro* and in vivo (Chen *et al.*, 1994; Iversen *et al.*, 1995). Two proteolytic cleavage events are required to generate Aβ from its precursor, one at the N-terminus by an enzyme termed β-secretase and one at the C-terminus by and enzyme termed γ-secretase. Among the secretases, β-secretase is at present the most attractive target for the inhibition of Aβ production. SR was proposed as a good candidate for β-secretase inhibitors (Lee *et al.*, 2005b). SR completely blocked Aβ (25-35)-induced neuronal cell death in the present study. These results imply the possibility of SR having neuroprotective effect in Alzheimer's brain with the prevention of the disease progression. SR has been known to contain glycosides and a large amount of tannic acid such as catechin and epicatechin ((Huang, 1999). We isolated various active components including gallic acid from SR. It, however, must be made further studies to clarify the active components to which SR-induced neuroprotection is attributable.

In conclusion, we demonstrated a novel pharmacological action of SR and its mechanism in the present study. The protection against Aβ (25-35)-induced neurotoxicity by SR may help to explain at least its inhibitory actions on the progression of AD, and furthermore provide the pharmacological basis of its clinical usage in treatment of neurodegeneration in AD.

## ACKNOWLEDGEMENTS

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## LITERATURE CITED

**Arias C, Arrieta I, Tapia R** (1995) β-Amyloid peptide fragment 25-35 potentiates the calcium-dependent release of excitatory amino acids from depolarized hippocampal slices. J. Neurosci. Res. 41:561-566.

**Ban JY, Seong YH** (2005) Blockade of 5-HT3 receptor with

MDL72222 and Y25130 reduces amyloid β protein (25-35)-induced neurotoxicity in cultured rat cortical neurons. Eur. J. Pharmacol. (in press).

**Behl C, Davis JB, Lesley R, Schubert D** (1994) Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77:817-827.

**Cardoso SM, Swerdlow RH, Oliveira CR** (2002) Induction of cytochrome c-mediated apoptosis by amyloid β 25-35 requires functional mitochondria. Brain Res. 931:117-125.

**Chen S-Y, Harding JW, Barnes CD** (1994) Neuropathology of synthetic β-amyloid peptide analogs in vivo. Brain Res. 715:44-50.

**Cheng DL, Cao XP** (1992) Pomolic acid derivatives from the root of Sanguisorba officinalis. Phytochemistry 31:1317-1320.

**Cotman CW, Pike CJ, Copani A** (1992) Beta-amyloid neurotoxicity: a discussion of *in vitro* findings. Neurobiol. Aging 13:587-590.

**Ekinci FJ, Linsley MD, Shea TB** (2000) β-Amyloid-induced calcium influx induces apoptosis in culture by oxidative stress rather than tau phosphorylation. Mol. Brain Res. 76:389-395.

**Ekinci FJ, Malik KU, Shea TB** (1999) Activation of the L voltage-sensitive calcium channel by mitogen-activated protein (MAP) kinase following exposure of neuronal cells to beta-amyloid. MAP kinase mediates β-amyloid-induced neurodegeneration. J. Biol. Chem. 274:30322-30327.

**Ellison DW, Beal MF, Martin JB** (1987) Amino acid neurotransmitters in postmortem human brain analyzed by high performance liquid chromatography with electrochemical detection. J. Neurosci. Methods 19:305-315.

**Ferger D, Krieglstein J** (1996) Determination of intracellular $Ca^{2+}$ concentration can be useful tool to predict neuronal damage and neuroprotective properties of drugs. Brain Res. 732:87-94.

**Forloni G** (1993) β-Amyloid neurotoxicity. Funct. Neurol. 8:211-225.

**Goodman Y, Mattson MP** (1994) Secreted forms of β-amyloid precursor protein protect hippocampal neurons against amyloid β-peptide-induced oxidative injury. Exp. Neurol. 128:1-12.

**Gray CW, Patel AJ** (1995) Neurodegeneration mediated by glutamate and peptide: a comparison and possible interaction. Brain Res. 691:169-179.

**Harkany T, Hortobagyi T, Sasvari M, Konya C, Penke B, Luiten PG, Nyakas C** (1999) Neuroprotective approaches in experimental models of β-amyloid neurotoxicity : relevance to Alzheimer's disease. Prog. Neuropsychopharmacol. Biol. Psychiatry 23:963-1008.

**Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen P, Wright K, Saad I, Mueller R, Morgan D, Sanders S** (1998) Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat. Med. 4:97-100.

**Horn J, Brouwers PJAM, Limburg M** (1999) Disturbances of calcium homeostasis in ischemic stroke: Therapeutical implication. CNS Drugs 11:373-386.

**Hsiao KH, Borchelt DR, Olson K, Johannsdottir R, Kitt C, Yunis W, Xu S, Eckman C, Younkin S, Price D, Iadecola C, Clark HB, Carlson G** (1995) Age-related CNS disorder and early death in transgenic FVB/N mice overexpressing Alzheimer amyloid precursor proteins. Neuron 15:1203-1208.

**Huang KC** (1999) The pharmacology of Chinese herbs, CRC press LLC, p. 155.

**Iversen LL, Mortishier-Smith RJ, Pollack SJ, Shearman MS** (1995) The toxicity *in vitro* of β-amyloid protein. Biochem. J. 311:1-16.

**Ivins KJ, Ivins JK, Sharp JP, Cotman CW** (1999) Multiple pathways of apoptosis in PC12 cells. CrmA inhibits apoptosis induced by beta-amyloid. J. Biol. Chem. 274:2107-2112.

**Koh JY, Yang LL, Cotman CW** (1990) β-Amyloid protein increases the vulnerability of cultured neurons to excitotoxic damage. Brain Res. 533:315-320.

**Lee BY, Ban JY, Seong YH** (2005a) Chronic stimulation of GABAA receptor with muscimol reduces amyloid β protein (25-35)-induced neurotoxicity in cultured rat cortical cells. Neurosci. Res. 52:347-356.

**Lee HJ, Seong YH, Bae KH, Kwon SH, Kwak HM, Nho SK, Kim KA, Hur JM, Lee KB, Kang YH, Song KS** (2005b) β-Secretase (BACE1) inhibitors from Sanguisorbae Radix. Arch. Pharm. Res. 28:799-803.

**Lee ZW, Kweon SM, Kim BC, Leem SH, Shin IC, Kim JH, Ha KS** (1998) Phosphatidic acid-induced elevation of intracellular $Ca^{2+}$ is mediated RhoA and H2O2 in rat-2 fibroblasts. J. Biol. Chem. 273:12710-12715.

**Li Q, Clark S, Lewis DV, Wilson WA** (2002) NMDA receptor antagonists disinhibit rat posterior cingulate and retrosplenial cortices: a potential mechanism of neu×Štoxicity. J. Neurosci. 22:3070-3080.

**Miranda S, Opazo C, Larrondo LF, Munoz FJ, Ruiz F, Leighton F, Inestrosa NC** (2000) The role of oxidative stress in the toxicity induced by amyloid β-peptide in Alzheimer's disease. Prog. Neurobiol. 62:633-648.

**Otto F, Tsou KC** (1985) A comparative study of DAPI, DIPI, and Hoechast 33258 and 33342 as chromosomal DNA stains. Stain Technol. 60(1):7-11.

**Pereira CF, Oliveira CR** (2000) Oxidative glutamate toxicity involves mitochondrial dysfunction and perturbation of intracellular $Ca^{2+}$ homeostasis. Neurosci. Res. 37:227-236.

**Shin TY, Lee KB, Kim SH** (2002) Anti-allergic effects of Sanguisorba officinalis on animal models of allergic reactions. Immunopharmacol. Immunotoxicol. 24:455-468.

**Tibor H, Maria S, Csaba K, Botond P, Paul GM, Luiten and Csaba N** (1999) Neuroprotective approaches in experimental models of β-amyloid neurotoxicity: relevance to Alzheimer's disease. Prog. Neuro. Psychopharmacol. 23:963-1008.

**Ueda K, Fukui Y, Kageyama H** (1994) Amyloid β protein-induced neuronal cell death: neurotoxic properties of aggregated amyloid β protein. Brain Res. 639:240-244.

**Ueda K, Shinohara S, Yagami T, Asakura K, Kawasaki K** (1997) Amyloid β protein potentiates $Ca^{2+}$ influx through L-

type voltage sensitive $Ca^{2+}$ channels : a possible involvement of free radicals. J. Neurochem. 68:265-271.

**Yan XT, Qiao JT, Dou Y, Qiao ZD** (1999) β-Amyloid peptide fragment 31-35 induces apoptosis in cultured cortical neu rons. Neuroscience 92:177-184.