Neuroprotective Properties of Gallic Acid from *Sanguisorbae* Radix on Amyloid β Protein (25—35)-Induced Toxicity in Cultured Rat Cortical Neurons

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Received August 14, 2007; accepted October 1, 2007; published online November 5, 2007

Our previous studies reported that methanol extract of *Sanguisorbae* radix from *Sanguisorba officinalis* L. (Rosaceae) prevented neuronal cell damage induced by Aβ (25—35) *in vitro*. The present study was carried out to investigate the effect of gallic acid isolated from *Sanguisorbae* radix on Aβ (25—35)-induced neurotoxicity using cultured rat cortical neurons. Gallic acid (0.1, 1 μM) showed a concentration-dependent inhibition on Aβ (25—35) (10 μM)-induced apoptotic neuronal death, as assessed by a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and Hoechst 33342 staining. Pretreatment of gallic acid inhibited 10 μM Aβ (25—35)-induced elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and generation of reactive oxygen species (ROS), which were measured by fluorescent dyes. Gallic acid also inhibited glutamate release into medium induced by 10 μM Aβ (25—35), which was measured by HPLC. These results suggest that gallic acid prevents Aβ (25—35)-induced apoptotic neuronal death by interfering with the increase of $[Ca^{2+}]_c$, and then by inhibiting glutamate release and generation of ROS, and that these effects of gallic acid may be partly associated with the neuroprotective effect of *Sanguisorbae* radix.

Key words gallic acid; *Sanguisorbae* radix; amyloid β protein; neuroprotection; cultured neuron

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cognitive impairment and progressive loss of memory. It is associated with the presence of intracellular neurobrillary tangles and extracellular amyloid deposits in the brain. The major constituent of amyloid deposits found in the plaques is amyloid β protein (Aβ), a 39—43 amino acid peptide derived from amyloid precursor protein.[1,2] Studies demonstrated *in vitro* and *in vivo* toxicities of Aβ or Aβ peptide fragments indicating an important role of Aβ in the pathogenesis of AD.[1,3,4] In cultures, Aβ can directly induce neuronal cell death and cause neurons vulnerable to excitotoxicity and oxidative insults.[3,5,6] It is suggested that the mechanisms underlying Aβ-neurotoxicity may involve the modulation of *N*-methyl-D-aspartate (NMDA) receptor, a glutamate receptor subtype, which can be caused by glutamate release, sustained elevations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and oxidative stresses.[5,7—10]

There has been much effort to develop beneficial agents from medicinal plants to achieve neuroprotection. Attention has been paid on a wide array of natural antioxidants that can scavenge free radicals and protect cells from oxidative damage such as resveratrol and catechins.[11,12] Gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, has been reported to show a number of biological activities. Gallic acid was found to induce apoptosis in tumor cells with higher sensitivity than normal cells.[13—15] This compound has also been described as an excellent free radical scavenger.[16] Due to this antioxidant effect, gallic acid containing plant extracts have showed the antidiabetic, antiangiogenic and antimelanogenic effects and reduced heart infarction incidence and oxidative liver and kidney damage.[17—21] Recently, gallic acid has been mentioned to protect Aβ-induced neuronal death in *in vitro*.[22] However, it remains undemonstrated how gallic acid exerts neuroprotection against Aβ-induced neuronal damage. In a previous study, we reported that methanol extract of *Sanguisorbae* radix, which has hemostatic, analgesic and astringent properties and has been used in traditional oriental medicine for a variety of disease,[23,24] protected Aβ (25—35)-induced neuronal cell damage in cultured rat cortical neurons.[25] We isolated two active principles, gallic acid and catechin, to which *Sanguisorbae* radix-induced neuroprotection might be attributable. The aim of the present study was to investigate the neuroprotective action of gallic acid against Aβ (25—35)-induced neuronal death, and the underlying mechanism in cultured rat cortical neurons.

MATERIALS AND METHODS

Plant Material, Extraction, Isolation, and Instrumental Analyses *Sanguisorbae* radix was purchased from an herbal market in Daegu, Korea and identified by professor K.-S. Song, Kyungpook National University. The voucher specimen (KNUNPC-SO-04-46) has been deposited at The Innovative Research Laboratory of Natural Products Medicine, Kyungpook National University, Daegu, Korea. The dried material (1 kg) was refluxed in 2 l methanol twice at room temperature. The methanol extract (150.23 g) was suspended in water and the suspension was consecutively partitioned with the same volume of dichloromethane, ethyl acetate (EtOAc), and *n*-butanol for the activity-guided purification. The EtOAc soluble fraction (9.75 g) was chromatographed on a Sephadex LH-20 column (3.6×68.4 cm, stepwise gradient of 10 to 100% methanol) to yield twelve

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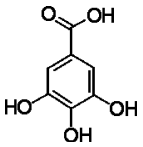

Fig. 1. Chemical Structure of Gallic Acid

fraction (Fr. 1—12). The Fr. 4 (480 mg) was re-chromatographed on a RP-C18 open column (1.5×40 cm, 50 to 80% methanol), followed by recrystallization in 50% methanol to give colorless needles (compound **1**, 50.0 mg). ^{1}H- and ^{13}C-NMR spectra were recorded on a UnityInova 500 spectrometer (Varian, U.S.A.) at 500 and 125 MHz, respectively and on a Bruker Avance Digital 400 spectrometer (Germany) at 400 MHz. Chemical shifts were given in δ (ppm) from TMS (tetramethylsilane). TLC was performed on a precoated silica gel plate (Merck, Art. 5715). Silica gel column chromatography was carried out using Kieselgel 60 (Art. 7734, Merck, Germany) and Sephadex LH-20 was supplied by Sigma (U.S.A.). Authentic gallic acid is a product of Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Compound **1**: Gallic acid: ^{1}H-NMR (methanol-d_4) δ: 7.06 (2H, s, H-2, H-3). ^{13}C-NMR (methanol-d_4) δ: 122.4 (C-1), 110.7 (C-2, 6), 146.8 (C-3, 5), 140.0 (C-4), 170.8 (C=O). The structure of gallic acid is presented in Fig. 1.

Chemicals (Reagents) Aβ (25—35) was purchased from Bachem (Bubendorf, Switzerland). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), *o*-phthaldialdehyde (OPA), 2-mercaptoethanol, gallic acid, trypsin (from bovine pancreas), Dulbecco's modified Eagle's medium (DMEM), Joklik-modified MEM, poly-L-lysine and amino acids for HPLC standard were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hoechst 33342 dye, fluo-4 AM and 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA) were purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). Fetal bovine serum was purchased from JRH Biosciences (Lenexa, KS, U.S.A.). All other chemicals used were of the highest grade available.

Experimental Animals Specific pathogen-free pregnant Sprague-Dawley (SD) rats (Daehan Biolink Co. Ltd., Chungbuk, Korea) were housed in an environmentally controlled room with temperature of 23±2 °C, relative humidity of 55±5%, and a 12-h light/dark cycle. Food and tap water for rats were available *ad libitum*. The procedures involving experimental animal comply with the regulations of the animal ethnical committee of Chungbuk National University for the care and use of laboratory animals.

Induction of Neurotoxicity in Primary Cultured Rat Cortical Neurons Primary cortical neuronal cultures were prepared using SD rat fetuses on embryonic day 15±1, according to the previously described methods.[26] Neurotoxicity experiments were performed on neurons grown for 5—6 d *in vitro*. Aβ (25—35) stock solution of 2 mM was prepared in sterile distilled water, stored at −20 °C, and incubated for more than 2 d at 37 °C to aggregate before use. Gallic acid was dissolved in methanol with the concentration of 10 mM and further diluted with experimental buffers. The final concentration of methanol was less than 0.1%, which did not affect cell viability. Neurons were treated with 10 μM Aβ (25—35) in serum-free growth medium for 24 h at 37 °C for the measurement of neuronal viability and reactive oxygen species (ROS) generation. For every experiment, gallic acid or its vehicle was applied 15 min prior to the treatment with Aβ (25—35) and was present in the medium during the incubation period with Aβ (25—35).

Analysis of Neuronal Viability Neuronal viability was monitored by the colorimetric MTT assay. The optical density was read at 570 nm using microplate reader (Bio-Tek EL_X808, Vermont, U.S.A.).[26] Chromatin condensation was detected by nucleus staining with Hoechst 33342. The nuclei of apoptotic cells were visualized under UV illumination using a fluorescence microscope (Olympus IX70-FL, Tokyo, Japan). 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 cells.[26]

Measurement of Aβ (25—35)-Induced Intracellular Biochemical Change The change of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was measured with fluo-4 AM, a fluorescent dye, using a laser scanning confocal microscope (Carl Zeiss LSM 510, Oberkochen, Germany).[26] Glutamate secreted into the medium for 6 h from Aβ (25—35) treated cells was quantified by high performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (BAS MF series, Indiana, U.S.A.).[26] The microfluorescence assay of 2′,7′-dichlorofluorescin (DCF), the fluorescent product of H_2DCF-DA, was used to monitor the generation of ROS. The neurons were observed by a laser scanning confocal microscope (Bio-Rad, MRC1024ES, Maylands, U.K.) using 488 nm excitation and 510 nm 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.[26]

Statistical Analysis Data were expressed as mean± S.E.M. and statistical significance was assessed by one-way analysis of variance (ANOVA) with subsequent Tukey's tests. p values of <0.05 were considered to be significant.

RESULTS

Gallic Acid Protects Neurons against Aβ (25—35)-Induced Cell Death The concentration of 10 μM was used for determining Aβ (25—35)-induced neuronal cell damage in the present experiments based on our previous results.[27,28] The inhibitory effect of gallic acid on Aβ (25—35)-induced neuronal apoptotic death of cultured cortical neurons was shown in Fig. 2. The treatment of cultured neurons with 10 μM Aβ (25—35) decreased MTT reduction rate to 69.6±3.1%. Gallic acid prevented the Aβ (25—35)-induced decrease of MTT reduction rate showing 93.4±1.2% with the concentration of 1 μM. On the other hand, a high concentration of gallic acid of 10 μM exerted a marked neuronal death showing 30.0±3.2% of MTT reduction rate, but neither protection nor exacerbation against 10 μM Aβ (25—35)-induced neurotoxicity (Fig. 2A). In a different experiment, the effect of gallic acid isolated from *Sanguisorbae* radix was compared to that of commercially available authentic gallic acid. The latter also prevented neuronal death showing 84.7±2.2% of cell viability with the concentration of 1 μM, when compared to 63.2±3.0% produced by 10 μM Aβ (25—35) (Fig. 2A). An additional experiment was performed with Hoechst 33342 staining to assess the neurotoxicity of Aβ

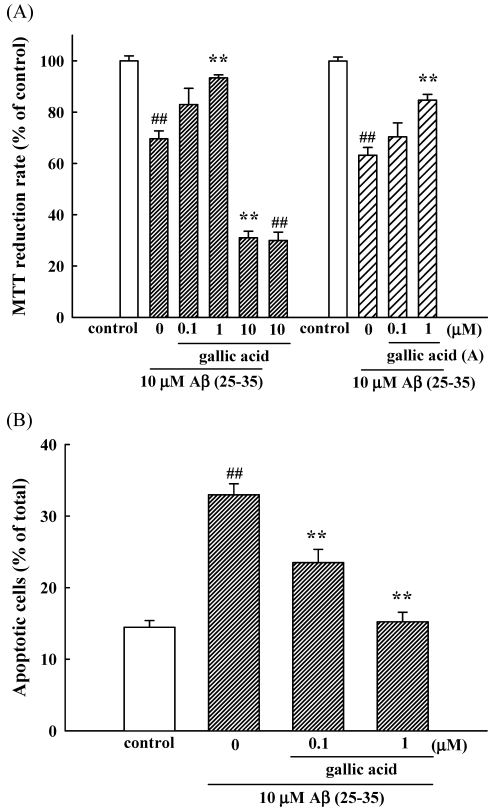

Fig. 2. Inhibitory Effect of Gallic Acid on Aβ (25—35)-Induced Apoptotic Neuronal Death in Cultured Cortical Neurons

(A) Neuronal death was measured by the MTT assay. The effect of authentic (A) gallic acid was compared that of gallic acid derived from *Sanguisorbae* radix. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean±S.E.M. values of the data obtained from three independent experiments performed in two to four wells. (B) Apoptotic cells measured by Hoechst 33342 staining were counted from 5 to 6 fields per well. Results are shown as apoptotic cells as a percentage of total number of cells and expressed as mean±S.E.M. values of the data obtained from at least three independent experiments performed in two to four wells. ## $p<0.01$ compared to control. ** $p<0.01$ compared to 10 μM Aβ (25—35).

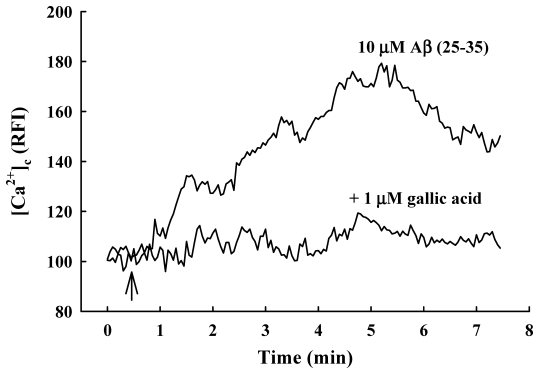

Fig. 3. Inhibitory Effect of Gallic Acid on Aβ (25—35)-$[Ca^{2+}]_c$ Elevation in Cultured Cortical Neurons

$[Ca^{2+}]_c$ was monitored using a laser scanning confocal microscope. All images from the scanning were processed to analyze changes of $[Ca^{2+}]_c$ in a single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative from at least three independent experiments.

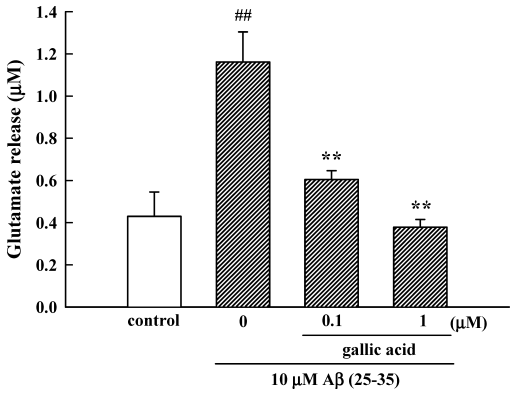

Fig. 4. Inhibitory Effect of Gallic Acid on Aβ (25—35)-Induced Glutamate Release in Cultured Cortical Neurons

The amount of released glutamate was measured by HPLC with ECD. Results are expressed as mean±S.E.M. values of the data obtained in three independent experiments performed in two wells. ## $p<0.01$ compared to control. ** $p<0.01$ compared to 10 μM Aβ (25—35).

(25—35). In Aβ (25—35)-treated neurons, chromatin condensation and nuclear fragmentation were observed, whereas the control culture had round blue nuclei of viable neurons. The treatment with 10 μM Aβ (25—35) produced apoptosis of 33.0±1.6% of the total population of cultured cortical neurons, when compared to 14.5±0.9% of apoptotic neurons in control cultures. The addition of gallic acid significantly decreased the Aβ (25—35)-induced apoptosis, showing 15.2±1.3% at the concentration of 1 μM (Fig. 2B). Low concentrations of gallic acid (0.1, 1 μM) did not affect cell viability (data not shown).

Gallic Acid 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 fluorescence 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 decreased gradually. In contrast, the treatment with 10 μM Aβ (25—35) in the presence of gallic acid (1 μM) failed to produce the elevation of $[Ca^{2+}]_c$ throughout the measurement period (Fig. 3). Gallic acid did not affect basal $[Ca^{2+}]_c$ (data not shown).

Gallic Acid Inhibits Aβ (25—35)-Induced Elevation of Glutamate Release Glutamate released into the extracellular medium was quantified 6 h after the incubation of neurons with 10 μM Aβ (25—35). As shown in Fig. 4, 10 μM Aβ (25—35) markedly elevated the basal glutamate level from 0.43±0.11 μM of control cells to 1.16±0.14 μM. Gallic acid (0.1, 1 μM) strongly blocked the Aβ (25—35)-induced elevation of glutamate release showing 0.60±0.04 μM and 0.38±0.04 μM, respectively.

Gallic Acid Inhibits Aβ (25—35)-Induced ROS Generation In 10 μM Aβ (25—35) treated neurons for 24 h, the fluorescence intensity increased about 5 folds to 121.7±7.1, indicating the increase of ROS generation, compared to control cells of 24.5±1.9. The Aβ (25—35)-induced increase of ROS generation was significantly inhibited by gallic acid (1 μM) showing 48.4±4.4 of fluorescence intensity (Fig. 5). Gallic acid did not show direct reaction with H_2DCF-DA to generate fluorescence (data not shown).

DISCUSSION

This study provides evidence that Aβ (25—35)-induced injury to rat cortical neurons was prevented by gallic acid

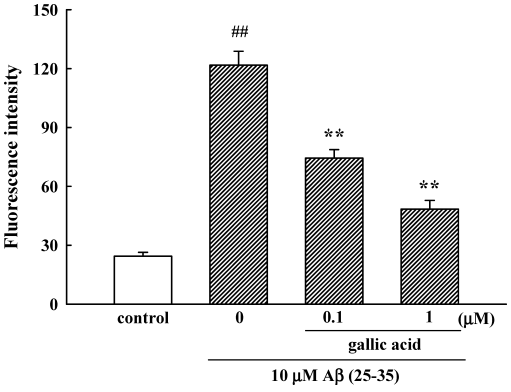

Fig. 5. Inhibitory Effect of Gallic Acid on Aβ (25—35)-Induced ROS Generation in Cultured Cortical Neurons

Values represent mean±S.E.M. of relative fluorescence intensity obtained from three independent experiments performed in two to five wells. ## $p<0.01$ compared to control. ** $p<0.01$ compared to 10 μM Aβ (25—35).

isolated from *Sanguisorbae* radix. This compound was able to reduce the Aβ (25—35)-induced $[Ca^{2+}]_c$ increase, glutamate release, ROS generation, and, in result, attenuate apoptotic neuronal death in primarily cultured rat cortical neurons. This result suggests that the neuroprotection of *Sanguisorbae* radix against Aβ (25—35)-induced neuronal cell damage may be, at least in part, attributable to gallic acid.

It has been demonstrated that Aβ-induced neurotoxicity may involve Ca^{2+} influx and generation of ROS.[3,7,8] In our previous studies, Aβ (25—35)-induced elevation of $[Ca^{2+}]_c$, glutamate release, ROS generation and then apoptotic neuronal cell death were blocked by the treatment with (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cycloheptene-5,10-imine (MK-801), an NMDA receptor antagonist, verapamil, an L-type Ca^{2+} channel blocker, and N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor.[27,28] These results suggest 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 proved by others.[4,5,7,10] It has been indicated that the primary event following Aβ (25—35) treatment of cultured neurons could 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.[29,30] 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 L-VDCC plays a critical role in the neurotoxicity.[27,28,31] In the present study, Aβ (25—35) elicited gradual and significant $[Ca^{2+}]_c$ increase, which was blocked by gallic acid. Gallic acid also significantly inhibited the Aβ (25—35)-induced glutamate elevation. These findings indicate that the sustained inhibition on $[Ca^{2+}]_c$ elevation by gallic acid resulted in the decrease of the Aβ (25—35)-induced glutamate release.

Many reports have demonstrated the role of ROS formation in Aβ-induced neurotoxicity.[5,10,30] Gallic acid decreased the Aβ (25—35)-induced increase of ROS generation in the present study. It has been reported that vitamin E, an antioxidant, blocked the Aβ-induced generation of ROS, but not Ca^{2+} influx. Moreover, a reduction of extracellular Ca^{2+} inhibited the Aβ-induced increase of $[Ca^{2+}]_i$ as well as ROS generation, indicating that ROS generation is a consequence of Ca^{2+} accumulation.[7,32] Furthermore, our previous studies demonstrated that the significant increase of ROS generation required at least 1 h, whereas the elevation of $[Ca^{2+}]_c$ occurred within seconds after the treatment with Aβ (25—35).[27] In addition, although L-NAME failed to inhibit the Aβ (25—35)-induced $[Ca^{2+}]_c$ increase during the short period of measurement, the complete inhibition of verapamil on the Aβ (25—35)-induced ROS generation was observed.[27,28] These reports together indicate that gallic acid inhibited the Aβ (25—35)-induced ROS generation *via* the blockade of $[Ca^{2+}]_c$ increase. Furthermore, 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, as described in other reports.[31,33] Gallic acid protected the neuronal cell against Aβ (25—35)-induced apoptotic death. It is thus concluded that gallic acid may prevent the Aβ (25—35)-induced apoptosis of neuronal cells by interfering with the increase of $[Ca^{2+}]_c$, and then by inhibiting glutamate release and ROS generation in cortical neurons. *In vivo* and *in vitro* studies have concluded that various beneficial effects of gallic acid and plants containing it were mainly due to its antioxidant effect.[17—21] Some gallic acid derivatives were demonstrated to have neuroprotective activity in 6-hydroxydopamine treated SH-SY5Y cells. Their neuroprotective properties depended on free radical scavenging effect.[34] A recent report also demonstrated the neuroprotection of gallic acid against Aβ (25—35)-induced toxicity in cultured hippocampal cells. They, however, focused on the inhibition on gallic acid-induced Aβ aggregation and/or formation of Aβ-derived diffusible neurotoxin ligands.[22] Therefore, to our knowledge, there are no reports on the direct modulation of gallic acid on the Aβ (25—35)-induced $[Ca^{2+}]_c$ change for the neuroprotection in cultured neurons. Aβ (25—35)-induced neurotoxic effects was prevented by inhibitors of endoplasmic reticulum (ER) Ca^{2+} release indicating the involvement of deregulation of ER Ca^{2+} homeostasis in neuronal death induced by Aβ.[35] In addition, the neuroprotective actions of some compounds and plant extracts in Aβ-induced toxicity have been shown to be involved in the inhibition of excess influx of Ca^{2+} in neuroblastoma cell lines and cultured neurons.[36,37] These reports suggest that neuronal loss induced by Aβ is due to the perturbation of intracellular Ca^{2+} homeostasis. Although the present study did not elucidate whether the inhibition by gallic acid on Aβ (25—35)-induced $[Ca^{2+}]_c$ elevation was due to inhibition on either Ca^{2+} influx or Ca^{2+} release from ER, we firstly show that gallic acid can protect neurons against Aβ-induced neurotoxicity, mainly *via* the inhibition of $[Ca^{2+}]_c$ elevation. Future study to clarify the precise mechanism should be performed.

On the other hand, gallic acid has been reported to induce apoptosis in various kinds of tumor cells. The increase of $[Ca^{2+}]_i$ and ROS generation and then caspase activation have been proposed as a major signaling pathway for the cytotoxic activity of gallic acid in tumor cell lines.[38,39] Gallic acid, indeed, produced a marked neuronal death without prevention of Aβ (25—35)-induced neuronal death of cultured cortical neurons at the concentrations of more than 10 μM in the present study. Moreover, 10 μM gallic acid and Aβ (25—35) did not produce an additive effect in neurotoxicity response. This

result indicates that higher concentration of gallic acid and Aβ (25—35) share a characteristic in terms of cytotoxicity involved in $[Ca^{2+}]_c$ increase and ROS generation.

Aβ is believed to play a central role in the pathophysiology of AD.[1—6] Gallic acid blocked Aβ (25—35)-induced neuronal cell death in present study. We isolated gallic acid and catechin from the methanolic extract of *Sanguisorbae* radix which was proved to inhibit Aβ (25—35)-induced neuronal damage in cultured cortical neurons.[25] In conclusion, gallic acid, together with catechin, which was also demonstrated to have neuroprotective effect against Aβ (25—35)-induced neurotoxicity,[40] could be responsible for the neuroprotective effect of *Sanguisorbae* radix. Furthermore we demonstrated a novel pharmacological action of gallic acid and its mechanism. The protection against Aβ (25—35)-induced neurotoxicity by gallic acid provides as a promising therapeutic approach to control the progression of neurodegeneration in brain of AD. Forthcoming studies will be attempted to clarify the *in vivo* effect of gallic acid.

Acknowledgements This work was supported by a grant (Code: 20050301 034398) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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