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Original Article

Neuroprotective Effects of Methanol Extract of *Sophorae Subprostratae Radix* on Glutamate Excitotoxicity in PC12 Cells and Organotypic Hippocampal Slice Cultures

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Objectives : It has been reported that *Sophorae Subprostratae Radix* (SSR) has a neuroprotective effect on cerebral ischemia in animals. In the present study, the authors investigated the neuroprotective effect of SSR on glutamate excitotoxicity. Glutamate excitotoxicity was induced by using NMDA, AMPA, and KA in PC12 cells and in organotypic hippocampal slice cultures.
Methods : Methanolic extract of SSR was added at 0.5, 5, and 50 μg/ml to culture media for 24 hours. The effects of SSR were evaluated by measuring of cell viability, PI-stained neuronal cell death, TUNEL-positive cells, and MAP-2 immunoreactivity.
Results : SSR increased PC12 cell viabilities significantly against AMPA-induced excitotoxicity, but not against NMDA-induced or KA-induced excitotoxicity. In organotypic hippocampal slice cultures damaged by NMDA-induced excitotoxicity, SSR attenuated neuronal cell death significantly in the CA1, CA3, and DG hippocampal regions and reduced TUNEL-positive cells significantly in CA1 and DG regions. In organotypic hippocampal slice cultures damaged by AMPA-induced excitotoxicity, SSR attenuated neuronal cell death and reduced TUNEL-positive cell numbers significantly in the CA1 and DG regions. In organotypic hippocampal slice cultures damaged by KA-induced excitotoxicity, SSR attenuated neuronal cell death significantly in CA3, but did not reduce TUNEL-positive cell numbers in CA1, CA3 or DG. In organotypic hippocampal slice cultures damaged by NMDA-induced excitotoxicity, SSR attenuated pyramidal neuron neurite retraction and degeneration in CA1.
Conclusions : These results suggest that the neuroprotective effects of SSR are related to antagonistic effects on the NMDA and AMPA receptors of neuronal cells damaged by excitotoxicity and ischemia.

Key Words : *Sophorae Subprostratae Radix*, hippocampal slice culture, propidium iodide (PI), TUNEL, MAP-2, PC12

Introduction

Glutamate is a type of excitatory neurotransmitter in the central nervous system and has an important role in the triggering of several functional and structural changes of neurons[1)]. On the other hand, excessive activation of glutamate receptors induces neuron neurodegeneration, a process called excitotoxicity, which is known to be an important injury mechanism in several ischemic brain diseases, e.g.,

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dementia, epilepsy, and several neurodegenerative diseases[2-4]. For example, synaptic glutamate is released during brain ischemia, and extracellular glutamate concentrations induce excessive intracellular Ca^{2+}, which consequently activates Ca^{2+} dependent proteases, lipases, and other modulators, and produces free radicals that kill neurons[3-6].

Sophorae Subprostratae Radix (SSR) induces detumescence, and relieves sore throats, and has been used as an oriental herbal medicine to treat seborrheic dermatitis, toxic heat, carbuncle in the throat, and lung cancer. It contains matrine, sophocarpine, and triterpenoid saponin[7-9] and has anticarcinogenic, anti-inflammatory, and antiulcer effects[10-13]. In addition, SSR is effective at treating neurological symptoms[14], and it has been reported that matrine reduces the brain edema that develops after brain ischemia by suppressing protein kinase C[15]. Furthermore, SSR has been reported to reduce areas and volumes of brain injury during the early stage of brain ischemia caused by middle cerebral artery occlusion, to suppress neuron injury at the cerebral cortex, and to suppress neuron atrophy[16].

Accordingly, the authors conducted the following experiment to elucidate the action mechanism of SSR. PC12 cells and organotypic hippocampal slice cultures were treated with the glutamate receptor agonist of N-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methyl-4- isoxazole propionic acid (AMPA) or kainic acid (KA) to induce excitotoxicity, and changes in cell survival rates, degrees of neuron death, and changes in neuron numbers showing a positive terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction were observed to determine the effect of SSR on neuron excitotoxicity by monitoring changes in neuritis by microtubule associated protein-2 (MAP-2) staining.

Materials and Methods

1. Preparation of the medication

SSR was purchased from the Department of Oriental Medicine, Oriental Hospital, Kyung Hee University Medical Center, ground and placed in a 5 L round-bottomed flask. A methanol/water mix (600 g; 70:30% v/v) was then added, and left for 5 days. The extract was filtered and vacuum evaporated using a rotary evaporator to remove the methanol completely, and the residue was freeze dried to yield 75.0 g of the methanolic extract of SSR, which was used in the following experiments.

2. Experiment on PC12 cell excitotoxicity

1) Cell culture and induction of excitotoxicity[17-19]

PC12 cells were placed in a 24-well plate at 1×10^6 cells per well in 500 ㎕ and incubated at 37℃ in a CO_2 incubator using a standard protocol. When cell growth reached the desired level, cells were washed with phosphate buffered saline (PBS), and culture medium was replenished with serum-free tissue culture medium. Excitotoxicity was induced by treating PC12 cells with the glutamate receptor agonists NMDA, AMPA, or KA at 10 μM, 3 μM or 8 μM, respectively.

2) Drug treatment and cell survival rate measurements

PC12 cells were treated with methanol extracts of SSR in at 0.5, 5 or 50 ㎍/ml for 1 hour and then incubated for 24 hours at 37℃ in a CO_2 incubator. They were treated with 3-(4,5-dimethyl- thiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT, 0.5 ㎎/㎖) and incubated for 2 hours. Culture media were then removed and 200 ㎕ of dimethyl sulfoxide was added and mixed. Absorbances were measured at 540 nm using an ELISA reader[20].

3. Experiment on excitotoxicity in organotypic hippocampal slice cultures

1) Hippocampal slice cultures

The hippocampus tissues used were obtained from 7-day-old Sprague-Dawley rats (Samtako, Seoul, Korea). The following procedures were used for sample preparation. Immediately after decapitation,

scalp and cranium were rapidly removed using an eye scissors to extract the cerebrum. With an extracted cerebrum immersed in anatomic media solution (MEM 500 ml + 25 mM HEPES + 4 mM L-glutamine, pH 7.4), left and right hemispheres were carefully separated under a surgical microscope and right and left hippocampal hemispheres was extracted. An extracted hippocampus was carefully placed on a McIlwain tissue chopper and tissue sections (350 ㎛) were prepared. Three hippocampus sections were placed on culture insert (Millipore, USA), and inserted into 6-well culture dish containing 1 ml of culture media, and incubated for 10 days at 36℃ in a 5% CO_2 humidified incubator[21,22].

Tissue culture medium was prepared using 50% MEM (containing 1g of glucose per 500 ml), 25% Hanks'balanced salt solution (HBSS), 20 nM HEPES, 6 g/L glucose, 1 mM L-glutamine, and 25% horse serum, filtered through a bottle top filter. pH was then adjusted to 7.25~7.30, and 50 mg/ml of streptomycin-penicillin added. The tissue culture medium was exchanged every 3 days during tissue culture.

2) Induction of excitotoxicity and treatment with medication[22,23]

To induce excitotoxicity in hippocampus tissue, NMDA, AMPA and KA were treated at 10 μM, 3 μM, and 8 μM, respectively, for 24 hours. Methanol extract of SSR was then added at 0.5, 5 or 50 ㎍/ml to each stage of culture media from 45 minutes before inducing excitotoxicity until 24 hours after excitotoxicity induction.

3) Propidium iodide staining and measurements of neuron death[24,25]

Hippocampal tissues were transferred into new 6-well plates containing 5 mg/ml of propidium iodide (PI) added to culture media, and preconditioned for 2 hours. Tissue culture was done by adding PI to each stage of culture media that was previously described above. Prior to SSR treatment and before inducing excitotoxicity, normal hippocampal tissue PI-stained images wereobtained by fluorescence microscopy (Axiovert S100, Zeiss). PI-stained images (A) were obtained 24 hours after inducing excitotoxicity. Tissues were treated with high concentrations of glutamate and PI-stained images of injured hippocampal tissues were obtained (B). PI-stained images were transferred to a computer image analyzer and red stained PI absorbance (optical density, OD) in the CA1 and DG regions were measured using NIH ImageJ software (Ver. 1.32). PI absorbance ratios of A/B were calculated to quantify neuron death.

4) TUNEL staining and positive neuron counts

NMDA, AMPA or KA were added to hippocampus slices, and then slices were fixed for 10 minutes with 1% paraformaldehyde containing PBS (pH 7.4), washed twice for 5 minutes, and refixed in ethanol-acetate solution at -20℃ for 20 minutes. The procedure of TUNEL staining involved the following:[26,27] slices were conditioned in 3% H_2O_2 in PBS for 5 minutes, treated with working strength TdT enzyme (70% reaction buffer + 30% TdT enzyme) at 37℃ for 2 hours, with stop/wash buffer for 15 minutes, with anti-digoxigenin peroxidase conjugatefor 1 hour, and with peroxidase substrate for 10 minutes (DAB dilution buffer + DAB substrate). Counter staining was practiced by treating sections with 0.5% methyl green for 5 minutes and washing with 100% n-butanol. Using NIH ImageJ software loaded into the computer image analyzer, CA1 and DG areas were measured, and neuron numbers showing TUNEL staining positivity were measured in these areas and expressed as counts per 10,000 $㎛^2$.

5) MAP-2 staining and observation[28,29]

After inducing excitotoxicity in hippocampal slices using NMDA, slices were fixed for 10 minutes with 1% PFA containing PBS buffer (pH 7.4), washed twice for 5 minutes, and refixed at 20℃ for 20 minutes with ethanol-acetate solution. The MAP-2 staining procedure used was as follows. Hippocampus slices were washed 3 times with 0.05 M PBS, reacted with 1% H_2O_2 for 10 minutes, washed, and reacted for 1 hour with a blocking solution that was

prepared by mixing 10% normal horse serum (Vectastain) and bovine serum albumin (Sigma) in PBS. Tissue slices were then rewashed and treated with primary antibody, anti-MAP-2 antibody (Sigma), which was diluted with PBS and Triton X-100 (1:200 dilution), and allowed to stand at 4℃ overnight. Sections were then washed with PBS, treated for 1 hour with avidin-biotin immunoperoxidase (ABC Vectastain Kit), and then with diaminobenzidine (Sigma). Sections were then attached to poly-L-lysine coated slides, dried for 2 hours, dehydrated, and then sealed. Changes in neurite in CA1 were observed under an optical microscope in the CA1 region, but neurite numbers were not quantified.

4. Statistical analysis

Analyses were performed using SPSS® for Windows (version 10.0, SPSS, Inc., Chicago). One-way ANOVA was used to compare expression levels at different SSR extract concentrations. Scheffe's test was performed as a post hoc test. P values of <0.05 were considered significant

Results

1. Effect of SSR extract on PC12 cell survival

When the survival rate of normal cells under normal conditions was set as 100%, NMDA treated cells had a mean survival rate of 83.4 ± 7.8%, and cells treated with 0.5 ㎍/ml of SSR extract had a survival rate of 73.0 ± 7.8%, with 5 ㎍/ml SSR extract a survival of 81.5 ± 7.9%, and 50 ㎍/ml SSR extract a survival of 70.9 ± 6.4%, respectively. However, treatment with SSR extract did not significantly affect survival. AMPA reduced survival to 57.1 ± 4.3%, and cells 0.5 ㎍/ml SSR extract a survival of 60.6 ± 6.6%, 5 ㎍/ml SSR extract 68.3 ± 4.3%, and 50 ㎍/ml SSR extract 71.2 ± 3.2%, respectively. Thus, SSR extract at 5 and 50 ㎍/ml significantly suppressed the cell death induced by AMPA (P<0.05). Similarly, treatment with KA reduced cell survival to 58.4 ±5.0%, but the treatment with SSR extract at 0.5 ㎍/ml increased cell survival to 63.0 ± 5.3%, 5 ㎍/ml of SSR extract to 65.3 ± 4.8%, and 50 ㎍/ml of SSR extract to 69.2 ± 5.5%, respectively, which showed that SSR extract did not have a significant effect on KA induced excitotoxicity in PC12 cells (Table 1).

2. Effect of the methanolic extract of SSR on neuron death by NMDA-induced excitotoxicity in organotypic hippocampal slice cultures

In the hippocampal slice cultures, neuron survival as determined by PI-absorbance ratios in normally cultured cells was limited in the CA1, CA3 and DG region (97.5 ± 0.7%, 98.2 ± 0.5% and 97.8 ±0.7%, respectively). In the NMDA treated group, cell survival rates in the CA1, CA3 and DG regions were 66.7 ± 5.4%, 37.9 ± 5.2% and 46.7 ± 4.7%, respectively. Cells treated with NMDA and 0.5㎍

Table 1. Effects of SSR extract on the viabilities of PC12 cells damaged by glutamate

(% of Normal)

Glutamate Receptor Agonists	Normal	Control	SSR(0.5)	SSR(5)	SSR(50)
NMDA	100.0	83.4 ± 7.8	73.0 ± 7.8	81.5 ± 7.9	70.9 ± 6.4
AMPA	100.0	57.1 ± 4.3	60.6 ± 6.6	68.3 ± 4.3*	71.2 ± 3.2*
KA	100.0	58.4 ± 5.0	63.0 ± 5.3	65.3 ± 4.8	69.2 ± 5.5

Data are presented as means ± standard error.
Normal: PC12 cells cultured under normal condition.
Control: PC12 cells cultured with glutamate receptor agonists (NMDA, 10 ㎛; AMPA, 3 ㎛; KA, 8 ㎛).
SSR(0.5): PC12 cells cultured with glutamate receptor agonists and 0.5 ㎍/ml of SSR extract.
SSR(5): PC12 cells cultured with glutamate receptor agonists and 5 ㎍/ml of SSR extract.
SSR(50): PC12 cells cultured with glutamate receptor agonists and 50 ㎍/ml of SSR extract.
: statistically significant compared to the control. (; P<0.05)

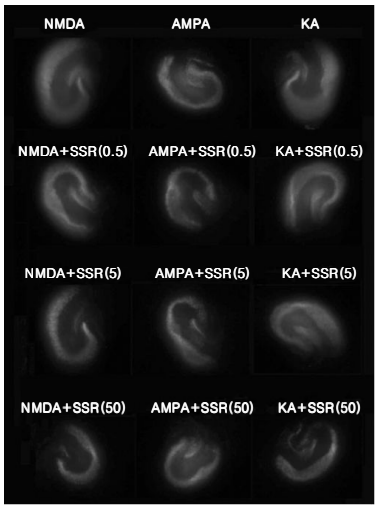

Fig. 1. Propidium iodide stained sections of organotypic hippocampal slice cultures. Red regions indicate neuronal cell death.

/ml of SSR extract had survivals in CA1, CA3 and DG of 61.3 ± 5.2%, 38.2 ± 5.2% and 41.5 ±5.4%, respectively. However, these results were not significantly different from those obtained in the NMDA group. When NMDA and 5 ㎍/ml of methanol extract of SSR were treated, we observed survivals in CA1, CA3 and DG of 49.0 ± 5.2%, 34.6 ± 4.0% and 34.5 ± 4.1%, respectively. Significant cell death suppression was observed in CA1 and DG ($P<0.05$, respectively). Cells treated with 50 ㎍/ml of SSR extract had survivals in CA1, CA3 and DG of 45.4 ±4.9%, 26.3 ± 3.9% and 31.3 ± 2.5%, respectively. SSR extract was found to have significant survival benefit in CA1, CA3, and DG ($P<0.01$ or $P<0.05$) (Fig. 1 and Fig. 2).

3. Effect of SSR extract on cell death induced by AMPA in the hippocampus

Neuron death survivals as determined by measuring PI-absorbance ratios in normally cultured group in CA1, CA3 and DG were almost 100%, i.e., 98.0 ± 0.8%, 98.7 ± 0.8% and 97.8 ± 0.7%, respectively. AMPA reduced cell survival in CA1, CA3 and DG to 50.1 ± 3.1%, 33.4 ± 2.8%, and 43.4 ± 3.2%, respectively. Slices treated with AMPA and 0.5㎍ /ml of SSR extract had cell survivals in CA1, CA3, and DG region of 43.0 ± 5.4%, 31.5 ± 4.1% and 41.5 ± 3.6%, respectively. These results were not significantly different than after AMPA-only treatment. When AMPA and 5 ㎍/ml of SSR extract were treated, cells survivals in CA1, CA3 and DG region were 37.3 ± 4.3%, 29.5 ± 4.7% and 35.0 ±5.1%, respectively. SSR extract had a significant prote-

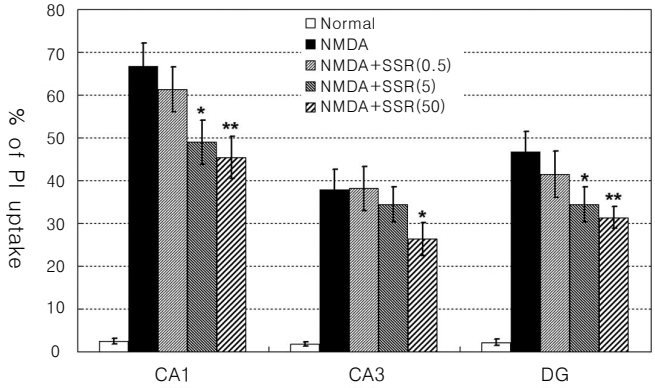

Fig. 2. Effects of SSR extract on neuronal cell death in hippocampal slice cultures damaged by NMDA-induced excitotoxicity.

SSR at 50 ㎍/ml significantly reduced neuronal cell death in the hippocampal CA1, CA3, and DG regions.
: statistical significance compared to the NMDA (, $P<0.05$; **, $P<0.01$).

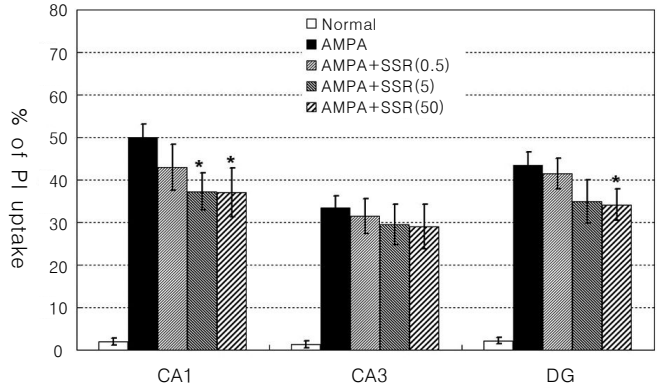

Fig. 3. Effects of SSR extract on neuronal cell death in hippocampal slices damaged by AMPA.
Treatment with SSR at 50 μg/ml significantly reduced neuronal cell death in the hippocampal CA1 and DG regions.
: statistical significance compared to the AMPA (, P<0.05).

ctive effect only in CA1 (P<0.05). Slices treated with AMPA and 50 μg/ml SSR extract had survivals in CA1, CA3, and DG of 37.0 ± 5.7%, 29.0 ± 5.3% and 34.2 ± 3.7%, respectively. Thus, SSR extract was found to have a significant protective effect on AMPA-induced excitotoxicity in CA1 and DG (P< 0.05, respectively) (Fig. 1 and Fig. 3).

4. Effect of SSR extract on PC12 cell death induced by the KA in organotypic hippoc-ampal slice cultures

Cell survivals determined by PI-absorbance ratios in normally cultured slices in CA1, CA3 and DG were hardly affected, i.e., 97.8 ± 0.5%, 98.3 ±0.8% and 97.8 ± 0.3%, respectively. However, survivals of cells treated with KA in CA1, CA3 and DG were significantly reduced to 28.4 ± 2.5%, 43.3 ± 3.4% and 35.1 ±3.0%, respectively. Cells treated with KA and 0.5μg/ml of SSR extract had survivals in CA1, CA3 and DG of 28.0 ± 3.9%, 36.5 ± 3.0% and 31.5 ± 4.7%, respectively. These results were

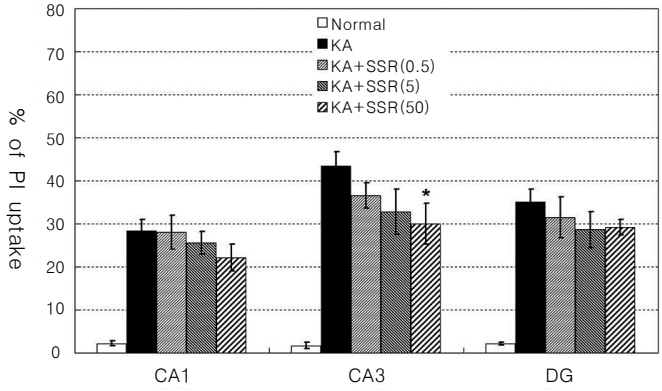

Fig. 4. Effects of SSR extract on neuronal cell death in hippocampal slices damaged by KA.
Treatment with SSR at 50 μg/ml significantly reduced neuronal cell death in the hippocampal CA3 region.
: statistical significance compared to the KA (, P<0.05).

not significantly different from those treated with KA alone. When KA and 5 ㎍/ml of SSR extract were treated, cells survivals in CA1, CA3 and DG were 25.6 ± 2.6%, 32.8 ± 5.2% and 28.6 ± 4.2%, respectively. These results were not significantly different from that of the KA group. When cells were treated with KA and 50 ㎍/ml of SSR extract mean cell survival rates in CA1, CA3 and DG reduction to 22.1 ± 3.1%, 30.0 ± 4.7% and 29.2 ±1.8%, respectively. These results show that SSR extract had a significant protective effect on the excitotoxicity induced by KA group in CA3 alone ($P<0.05$) (Fig. 1 and Fig. 4).

5. Effect of SSR extract on TUNEL-positivity nerve counts after NMDA challenge in hippocampal slice cultures

Neuron counts for TUNEL-positivity after NMDA treatment showed 50.1 ± 4.4, 27.9 ± 3.0 and 39.6 ± 2.7 per 10,000 ㎛^{2} in the CA1, CA3 and DG, respectively. Slices treated with NMDA and 50 ㎍/ml of SSR extract showed significant neuron count reductions to 33.7 ± 3.5, 24.7 ± 2.7 and 31.7 ± 2.4 per 10,000 ㎛^{2} in CA1, CA3 and DG, respectively. Significantly lower neuron TUNEL-positive count were observed in CA1 and DG in SSR-treated groups than in the NMDA group ($P<0.01$ and $P<0.05$, respectively) (Table 2, Fig. 5).

6. Effect of SSR extract on TUNEL-positive neuron counts induced by AMPA hippocampal slice cultures

TUNEL-positive neuron counts induced by AMPA in hippocampal slice cultures were 36.7 ± 4.0, 26.7 ± 3.6 and 33.4 ± 3.6 per 10,000 ㎛^{2} in CA1, CA3, and DG, respectively. Slices treated with AMPA and 50 ㎍/ml of SSR extract showed significant cell reductions to 27.5 ± 2.9, 26.2 ± 4.2 and 24.5 ± 3.2 per 10,000 ㎛^{2} in CA1, CA3 and DG, respectively. A significant reduction in TUNEL-positive neuron count in CA1 and DG was observed in SSR treated slices ($P<0.05$, respectively) (Table 2, Fig. 5).

7. Effect of SSR extract on TUNEL positive neuron counts induced by KA-induced excitotoxicity in hippocampal slice cultures

The numbers of TUNEL-positive cells induced by KA in hippocampal slices were 24.4 ± 3.1, 41.7 ± 3.3 and 31.7 ± 4.0 per 10,000 ㎛^{2} in CA1, CA3, and DG, respectively. Slices treated with KA and 50 ㎍/ml of SSR extract showed significant neuron count reductions to 22.0 ± 3.1, 33.3 ± 4.0 and 27.5

Table 2. Effects of SSR extract on TUNEL-positive Cells in hippocampal slice cultures damaged by NMDA, AMPA or KA (Number of Cells/10,000 ㎛^{2})

Groups	Regions in Hippocampus		
	CA1	CA3	DG
NMDA	50.1 ± 4.4	27.9 ± 3.0	39.6 ± 2.7
NMDA+SSR(50)	33.7 ± 3.5**	24.7 ± 2.7	31.7 ± 2.4*
AMPA	36.7 ± 4.0	26.7 ± 3.6	33.4 ± 3.6
AMPA+SSR(50)	27.5 ± 2.9*	26.2 ± 4.2	24.5 ± 3.2*
KA	24.4 ± 3.1	41.7 ± 3.3	31.7 ± 4.0
KA+SSR(50)	22.0 ± 3.1	33.3 ± 4.0	27.5 ± 3.3

Data are presented means ± standard error.
NMDA: organotypic hippocampal slices cultured with 10 μM of NMDA.
NMDA+SSR(50): organotypic hippocampal slices cultured with NMDA and 50 μg/ml SSR extract.
AMPA: hippocampal slices cultured with 3 μM of AMPA.
AMPA+SSR(50): hippocampal slices cultured with AMPA and 50 μg/ml of SSR extract.
KA: hippocampal slices cultured with 8 μM of KA.
KA+SSR(50): hippocampal slices cultured with KA and 50 μg/ml of SSR extract.
: statistical significance compared to NMDA treatment. (, $P<0.05$; **, $P<0.01$)

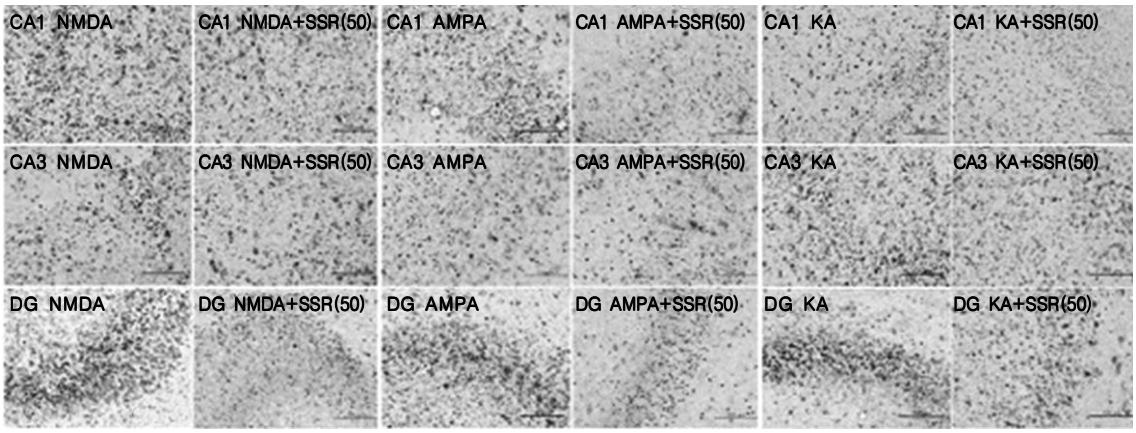

Fig. 5. TUNEL immuno-stained sections of hippocampal slices damaged by NMDA, AMPA or KA.
SSR treatment at 50 μg/ml significantly reduced TUNEL-positive cell numbers in the hippocampal CA1 and DG regions.

± 3.3 per 10,000 μm^2 in CA1, CA3 and DG, respectively. No significant reduction of neuron count that showed TUNEL-positivity was observed compared to the AMPA group (Table 2, Fig. 5).

8. Effect of SSR extract on neurites damage induced by NMDA hippocampal slices

Under normal culture conditions, the neurites of pyramidal neurons stained by MAP-2 inCA1 revealed a complex dense structure. However, in slices treated with NMDA nearly all neurites of pyramidal neurons appeared shrunken or degenerated. In slices treated with NMDA and 5 or 50 μg/ml of SSR extract revealed to show significant reduction of neurites of pyramidal neuron in CA1 region compared to the NMDA group, and wholesome pyramidal neurons could be observed (Fig. 6).

Discussion

In a previous study[16], SSR was found to significantly reduce brain ischemia injured areas and volumes caused by middle cerebral artery occlusion, to significantly reduce neuron injury in the cerebral

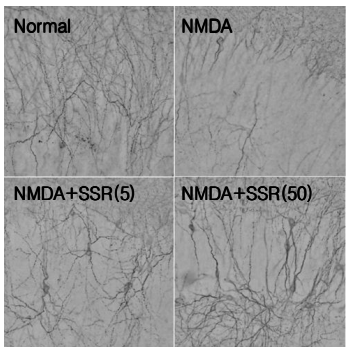

Fig. 6. MAP-2 immuno-stained sections of hippocampal slices damaged by NMDA.
Treatment with SSR at 5 or 50 μg/ml significantly increased numbers of pyramidal neuron neurites in the hippocampal CA1 region.

cortex, and to significantly suppress the effect of neuron atrophy. In the present study, we undertook to further investigate the mechanism underlying the effect of SSR extract on brain ischemia.

Neurotransmitters facilitate signal transmission at synapses in the mammal brain, and glutamate plays an important role in the normal neuron function and development[1]. However, epilepsy, brain damage, and brain ischemia may induce excessive amounts of glutamate in the medium around neurons. Furthermore, localized edema is also induced, which contributes to neurite degeneration and neuron death[2,4]. This effect of glutamate involves three types of ionotrophic receptors located at neuronal membranes, which act at NMDA (N-methyl-D-aspartate), AMPA (2-amino-3-hydroxy-5- methyl-4-isoxazole propionic acid), or KA (kainic acid) receptors. The excitotoxicities of glutamate and of the glutamate receptor agonists NMDA, AMPA, and KA have been well established by many in vivo and *in vitro* studies, and it is well known that excessive glutamate receptor activity is the most important mechanistic contributor to neurodegenerative disease and environment induction[3,5,30,31]. In the present study, the glutamate receptor agonists NMDA, AMPA, and KA were separately administered to PC12 cells and to hippocampal slices, and these effect of SSR extract on the excitotoxic effects of theseagents were examined to determine whether SSR extract directly affects glutamate receptors.

PC12 cells (a neural progenitor cell-line differentiated from neural crest cells) were treated with NMDA, AMPA, or KA to induce excitotoxicity and the methanol extract of SSR was treated at 0.5, 5 and 50 μg/ml to observe its effect on cell survival. It was found that the methanol extract of SSR did not have a significant effect on NMDA- or KA-induced excitotoxicity, but that it had a significant protective effect at 5 or 50 μg/ml on AMPA-induced excitotoxicity. This result indicates that the neuroprotective effect of SSR extract involves blocking AMPA receptors.

The hippocampus is the part of the brain that reacts most sensitively to ischemic injuries and that is most vulnerable to neuronal damage. Since the hippocampus tissue culture protocol reflects the in vivo condition, it has many advantages over *in vitro* experiments[21,22]. Furthermore, this technique allows substances like NMDA, AMPA and KA to be treated at different concentrations to induce excitotoxicity, and allowed us to treat sections with SSR extract to observe neuron death and changes in TUNEL positive neuron counts. In terms of the induction of excitotoxicity, NMDA, AMPA, and KA have different efficacies. Therefore, NMDA, AMPA, and KA treatments were made at concentrations that cause 50% neuron death in organotypic hippocampal slice cultures over 24 hours. Accordingly, NMDA was treated at 10 μM, AMPA at 3 μM, and KA at 8 μM. Neuron deaths were determined by measuring PI-absorbance ratios using the PI fluorescence staining method. Because cell membranes are damaged during the cell death process, PI penetrates cells, attaches to DNA in nuclei, and produces fluorescence[24,25]. In addition, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method is a frequently used histological method to determine natural cell death[26,27]. This method relies on DNA cleavage in nuclei which occurs during the course of natural cellular death.

In terms of the excitotoxic effect of NMDA in hippocampal slice cultures, 5 and 50 μg/ml of SSR extract were found to significantly suppress neuron death in CA1, CA3, and DG. In addition, SSR also significantly suppressed TUNEL positive neuron counts in CA1 and DG. These results explain the suppressive effect of SSR extract on the neuron death caused by NMDA. Furthermore, these results indicate that the neuroprotective effect of SSR extract is achieved by a mechanism that blocks NMDA receptors. On the other hand, this result contradicts the finding that SSR extract did not have a significant effect on NMDA-induced excitotoxicity in PC12 cells, which we attribute to different experimental procedures. This result appears to be attributable to the characteristics of PC12 cells, in

view of the fact that it has been reported that NMDA receptor blockers do not significantly suppress PC12 cell death, and that NMDA treatment alone does not properly induce excitotoxicity[32,33].

In the case of AMPA treatment to hippocampal slices, 50 ㎍/ml of SSR extract was found to significantly suppress neuron death in CA1 and DG, and to significantly reduce TUNEL-positive neuron numbers. These findings demonstrate that SSR extract has a suppressive effect on neuron death caused by AMPA, which indicates that the neuroprotective effect of SSR extract is dependent on an AMPA receptor blockade. In addition, SSR extract produced similar results in PC12 cells and in hippocampal slice cultures.

In terms of the excitotoxic effect of KA on slice cultures, SSR extract at 50 ㎍/ml significantly suppressed neuron death only in CA3. However, SSR extract failed to reduce TUNEL-positive neuron counts in the other hippocampal regions at any concentration. This is probably due to the characteristics of KA, which selectively damages pyramidal neurons in CA3, unlike NMDA or AMPA[23,34,35].

In cases of brain ischemia or brain injury, neuron neurites shrink or degenerate, which causes signal transmission problems in synapses even if neuron death has not occurred. On the other hand, neurite shrinkage and degeneration have been reported to occur prior to neuron death[28,29], and both in vivo and *in vitro* experiments have shown that glutamate, NMDA, and AMPA cause neurite shrinkage and degeneration[36,37]. Therefore, in the present study, SSR extract was treated at 5 and 50 ㎍/ml after inducing excitotoxicity in hippocampal slices using NMDA. Furthermore, neurites were selectively immunostained using MAP-2 to observe neurite change in pyramidal neurons. As a result, pyramidal neurons stained with MAP-2 in normal hippocampal slice cultures revealed complex and dense neurite staining in CA1 region, and changes in neurites induced by NMDA in slices showed that nearly all neurites were atrophied or degenerated. In addition, SSR extract at 5 and 50 ㎍/ml significantly reduced neurite atrophy and degeneration induced by NMDA.

Summarizing the results of the present study, SSR extract was found to significantly suppress the neuron death, neurite atrophy and degeneration caused by glutamate-induced excitotoxicity. This effect of SSR extract and its neuroprotective effects on neurons after ischemia are considered to be mechanisms that involve antagonistic responses to NMDA and AMPA receptors, which are responsible for glutamate-induced excitotoxicity in neurons.

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References

1. Kuh JS, Kim JH. Effects of *Chungpyesagantang* on the Diabetic Rats induced by Streptozotocin. The Journal of Korean Oriental Pediatrics. 1997; 11(1):227-48.
2. Heo SY, Kang HS. Experimental study on the effect of *Chungpyesagantan* on obese rats. Journal of Oriental Rehabilitation Medicine. 1998;8(2): 106-26.
3. Nicholson DW. Mechanisms of apoptotic control. Nature. 2000;407:810-6.
4. Laake JH, Haug FM, Wieloch T, Ottersen OP. A simple *in vitro* model of ischemia based on hippocampal slice cultures and propidium iodide fluorescence. Brain Res. Protoc. 1999;4:173-84.
5. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol. 1992;119:493-501.
6. Dong LP, Wang TY. Effects of puerarin against glutamate excitotoxicity on cultured mouse cerebral cortical neurons. Zhongguo Yao Li Xue

Bao. 1998;19(4):339-42.
7. Lee JM. Dongeuisusebowon. Seoul: Haenglimse-owon. 1986:7-13, 115, 137-138.
8. Kwon D, Goh CN, Joh G, Gi YS, Bae HS, Lee KS. Clinical Observation on the Admitted Patients of Department of Cardiac Internal Medicine of Oriental Hospital. The Journal of Kyung Hee University Medical Center. 1996;12(2):200-13.
9. Kristensen BW, Noraberg J, Zimmer J. Comparison of excitotoxic profiles of ATPA, AMPA, KA and NMDA in organotypic hippocampal slice cultures. Brain Res. 2001;917:21-4.
10. Kim DR, Koh BH, Song IB. An experimental study of the effect by *ch'ongshinyonjat'ang* and *ch'ongpyesangant'ang* on immune response and anti-allergic reaction to rats and mice. Journals collection of Kyoung Hee University. 1991;14: 131-60.
11. Song IY, Lee JS, Kim SS, Shin HD. Effect of *Chungpyesagan-Tang* and *Taeumjowui-Tang* on soft mass during fasting therapy. Journal of Oriental Rehabilitation Medicine. 1998;8(1):34-56.
12. Kroemer G, Reed J. Mitochondrial control of cell death. Nat. Med. 2000;6:513-9.
13. Bonde C, Sarup A, Schousboe A, Gegelashvili G, Noraberg J, Zimmer J. GDNF pre-treatment aggravates neuronal cell loss in oxygen-glucose deprived hippocampal slice cultures: a possible effect of glutamate transporter up-regulation. Neurochem. Int. 2003;43(4-5):381-8.
14. Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotkovsky B, Orrenius S, Lipton SA, Nicotera P. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron. 1995;15:961-73.
15. Brana C, Benham C, Sundstrom L. A method for characterizing cell death *in vitro* by combining propidium iodide staining with immunohistochemistry. Brain Res. Protoc. 2002;10:109-14.
16. Bae CH, Joh GH, Lee WC, Kim YS, Bae HS, Lee KS, Khu BH. Clinical observation on C.V.A. Journal's collection of Kyoung Hee University. 1987;10:665-87.
17. Kim JJ, Kim HJ, Ahn BK, Hong WS, Lee SG, Kim WH, Lee HI, Ahn DK, Shin MK. Study for herbal remedy on Liver disease (II) Experimental study for effect of *Chungpyesagantan*. Journals collection of Kyoung Hee University. 1978;1:9-14.
18. Lobner D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis. J Neurosci. Methods. 2000; 96:147-52.
19. Ahlemeyer B, Klumpp S, Krieglstein J. Release of cytochrome c into the extracellular space contributes to neuronal apoptosis induced by staurosporine. Brain Res. 2002;934:107-16.
20. Tian J, Du H, Yang H, Liu X, Li Z. A clinical study on compound *da huang (radix et rhizoma rhei)* preparations for improvement of senile persons' memory ability. J Tradit. Chin. Med. 1997;17(3):168-73.
21. Hilton DA, Love S, Barber R. Demonstration of apoptotic cells in tissue sections by *in situ* hybridization using digoxigenin-labeled poly(A) oligonucleotide probes to detect thymidine-rich DNA sequences. J Histochem. Cytochem. 1997; 45:13-20.
22. Love S, Hilton DA. Comparison of methods for the detection of cells in different stages of apoptosis. Neuropathol. Appl. Neurobiol. 1996;22: 161-2.
23. Hengartner M. The biochemistry of apoptosis. Nature. 2000;407:770-7.
24. Kim DG,Ryu KW. Effects of *Chungpesagan-Tang* on the Liver and Kidney Dysfunction due to Mercuric Chloride Poisoning in Rabbits. The Journal of Korean Oriental Medicine. 1983;4 (1):3-29.

25. Hwang YS, Shin CY, Huh Y, Ryu JH.Hwangryun-Hae-Dok-tang (Huanglian-Jie-Du-Tang) extract and its constituents reduce ischemia-reperfusion brain injury and neutrophil infiltration in rats. Life Sci. 2002;71(18):2105-17.
26. Zimmer J, Gahwiler BH. Cellular and connective organization of slice cultures of the rat hippocampus and fascia dentata. J Comp. Neurol. 1984; 228:432-46.
27. Noraberg J, Kristensen BW, Zimmer J. Markers for neuronal degeneration in organotypic slice cultures. Brain Res. Protoc. 1999;3:278-90.
28. Hong SG, Kang BG, Kim YJ, Joh DW, Kang SM. Protective effects of *Chungpesagan-tang* against ischemia/reperfusion induced cell injury. Korean Journal of Oriental Medicine . 2000;5(1): 111-17.
29. Wang L, Zhao A, Wang F, Chai Q, Chai X. Protective effect of puerarin on acute cerebral ischemia in rats. Zhongguo Zhong YaoZa Zhi. 1997;22(12):752-4.
30. Jang MH, Shin MC, Chung JH, Shin HD, Kim Y, Kim EH, Kim CJ. Effects of Puerariae radix on cell proliferation and nitric oxide synthase expression in dentate gyrus of alcohol-intoxicated Sprague-Dawley rats. Jpn. J Pharmacol. 2002;88 (3):355-8.
31. Wilde GJC, Sundstrom LE, Iannotti F. Propidium iodide in vivo: an early marker of neuronal damage in rat hippocampus. Neurosci Lett. 1994; 18:223-6.
32. Oh YH,Jeong SH,IS,GC,e WC. The Effects of *Chungpesagan-Tang* Extracts on Reversible Forebrain Ischemia in Sprague-Dawley Rats. The Journal of Korean Oriental Internal Medicine. 2001;22(2):161-74.
33. Monette R, Small DL, Mealing G, Morley P. A fluorescence confocal assay to assess neuronal viability in brain slices. Brain Res. Protoc. 1998; 2:99-108.
34. Xu GP, Dave KR, Vivero R, Schmidt-Kastner R, Sick TJ, Porez-Pinzon MA. Improvement in neuronal survival after ischemic preconditioning in hippocampal slice cultures. Brain Res. 2002; 952:153-8.
35. Ahn DK,DH, Kim JH. Neuroprotective Effects of the Rhei Rhizoma on the Rats' Transient Forebrain Ischemia caused by 4-vessel-occlusion. The Korea Journal of Herbology. 1999;14(1): 111-20.
36. Bonde C, Noraberg J, Zimmer J. Nuclear shrinkage and other markers of neuronal cell death after oxygen-glucose deprivation in rat hippocampal slice cultures. Neurosci. Letter. 2002;327:49-52.
37. Gahwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM. Organotypic slice cultures: a technique has come of age. Trends Neurosci. 1997;20:471-7.