

Protection of spontaneous and glutamate-induced neuronal damages by Soeumin Sibjeundaibo-tang and Soyangin Sibimijihwang-tang in cultured mice cerebrocortical cells

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Running title: Protection of neuronal damage by herbal medicines.

SUMMARY

Soeumin Sibjeundaibo-tang (SJDBT) and Soyangin Sibimijihwang-tang (SMJHT) have been used traditionally to improve the systemic blood circulation and biological energy production in the patients with circulatory and neuronal diseases. The object of this study is to determine the protective effects of SJDBT and SMJHT extracts on the spontaneous and glutamate-induced neuronal damages in cultured cells derived from mice cerebral cortex. At 14 days after beginning the cultures, the activity of lactate dehydrogenase released into the culture media was significantly decreased by treatment of cerebrouneural cells with SJDBT and SMJHT (0.1 mg/ml) for 7 days. By comparison with the normal cells, cerebrouneural morphology was dramatically changed by treatment of glutamate (1 mM) for 12 hrs, and this was conspicuously recovered by pretreatment of cerebrouneural cells with SJDBT and SMJHT (0.1-1.0 mg/ml) for 2 days. Moreover, glutamated-induced DNA fragmentation was also protected by pretreatment of cerebrouneural cells with those extracts. These results suggest that naturally occurring and glutamate-induced degeneration of cultured cerebrocortical cells may be related, in part, to the process of apoptotic cell death. The pharmacological properties of SJDBT and SMJHT extracts to improve cerebrouneural degeneration may be considered as one of useful medicines that can prevent cerebrocortical impairments resulted from age-dependent and excitotoxicity-induced neuronal degeneration in human brain.

Key words: Soeumin Sibjeundaibo-tang; Soyangin Sibimijihwang-tang; Cerebral cortex; Neurodegeneration; Cultured cells.

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INTRODUCTION

Necrosis and apoptosis are distinct mechanisms of cells death with very different

characteristics. During development and maturation in the nervous system, a large excess of neurons is produced and subsequently eliminated to maintain neuronal

homeostasis (Blaschke *et al.*, 1996; Lucassen *et al.*, 1995). This neuronal pruning is mediated mainly by apoptosis, also referred to as programmed cell death, which often involves characteristic morphological alteration, DNA fragmentation and de novo RNA and protein synthesis (Oppenheim *et al.*, 1990; Martin *et al.*, 1988). In the mode of neuronal cell death in vitro, Ishitani *et al.* (1996a and 1996b) suggested that mature neuronal cells undergo an age-induced apoptotic death in culture. Therefore, it is generally considered that naturally occurring cell death in the developing and matured neuronal cells is a widely distributed phenomenon, and has the morphology of apoptosis which is associated with endonuclease activation (Ferrer *et al.*, 1994).

On the other hand, Soeumin Sibjeundaibotang (SJDBT) and Soyangin Sibimijihwangtang (SMJHT) are noted prescriptions among traditional al medicines in Korea, and have been widely used for a variety of diseases such as stroke, palsy and amnesia in elderly patients (Lee, 1986; Lee and Hong, 1992). Regarding in their traditional use, these prescriptions have been considered to enhance systemic blood circulation and biological energy production in the patients with neuronal and circulatory diseases. In support of this traditional hypothesis, recent report suggested that SJDBT might participate in improvement of declined energy production and cholinergic neurotransmitter synthesis in the mice cerebral cortex with memory-loss (Ma *et al.*, 1999). However, cellular mechanism of SJDBT and SMJHT on the brain associated with cerebneurodegeneration has not been well understood. An evaluation of these mechanisms may play an important role in the improvement of age-dependent neurological disorders. Regarding in these, a primary object of the current study is to determine whether SJDBT and SMJHT affect the naturally occurring and glutamate-induced neuronal degeneration in the cultured

cerebrocortical cells derived from mice brain.

MATERIALS AND METHODS

Cell culture

Primary cerebrocortical cultures were prepared from 1-day-old mice, essentially according to the dissociation procedures described in the previous reports (Hatten, 1985; Ishitani *et al.*, 1996a; Takeshima *et al.*, 1994). Briefly, after careful dissociation from diencephalic structures and hippocampus, the cerebral cortices of 8 to 10 mice were pooled and sliced (0.4 mm in thickness) in two orthogonal directions. The cubes were incubated in a 0.025% trypsin solution and dispersed by trituration in a solution containing DNase (0.01%) and soybean trypsin inhibitor (0.05%). Cells (1×10^6 cells/dish) were plated into 35 mm Primaria plate (Becton Dickinson, Lincoln Park, NJ) and were cultured at 37°C in a humidified 5% CO₂ atmosphere in growth medium containing Dulbecco's modified Eagle's medium, 10% inactivated fetal bovine serum, 100U/ml penicillin and 10 µg/ml streptomycin. After 2 days, cytosine arabinoside (5mM) was added to the media for 24 hrs to inhibit the replication of non-neuronal cells (Dessi *et al.*, 1995). The medium was changed every 3-4 days.

Preparation of herbal extracts

All plant materials used in this study were purchased from the Korea Medicine Herbs Association and from the most famous cultivating districts in Korea and authenticated by both botanists and pharmacognosists at the Korea Institute of Oriental Medicine. Their voucher specimens (No. KIOM 97-3-7 and 97-3-9) had been deposited at the herbarium at the Korea Institute of Oriental Medicine. The ingredients (41.25g) of SJDBT consisted of Ginseng radix (7.5g), Astragali radix (3.75g), *Atractylodis macrocephalae*

rhizoma (3.75g), *Paeoniae radix* (3.75g), *Angelicae gigantis radix* (3.75g), *Cnidii rhizoma* (3.75g), *Citri pericarpium* (3.75g), *Glycyrrhizae radix* (3.75g), *Polygoni multiflori radix* (3.75g) and *Cinnamomi cortex* (3.75g). These ingredients correspond to parts of the following plants: *Panax ginseng* C. A. Meyer (Araliaceae), *Astragalus membranaceus* Bunge (Leguminosae), *Atractylodes japonica* Koidzumi (Compositae), *Paeonia albiflora* Pall var. *tricarpa* Bunge (Ranunculaceae), *Angelica gigas* Nakai (Umbelliferae), *Cnidium officinale* Makino (Umbelliferae), *Citrus unshiu* Markovich (Rutaceae), *Glycyrrhiza uralensis* Fischer (Leguminosae), *Polygonum multiflorum* Thunberg (Polygonaceae) and *Cinnamomum cassia* Blume (Lauraceae), respectively. The ingredients (58.125g) of SMJHT consisted of *Rehmanniae radix preparata* (15.0g), *Corni fructus* (7.5g), *Poria* (5.625g), *Alismatis rhizoma* (5.625g), *Moutan cortex* (3.75g), *Lycii radices cortex* (3.75g), *Scrophulariae radix* (3.75g), *Lycii fructus* (3.75g), *Rubi fructus* (3.75g), *Plantaginis semen* (3.75g), *Schizonepetae herba* (3.75g) and *Ledebouriellae radix* (3.75g). These ingredients correspond to parts of the following plants: *Rehmannia glutinosa* Liboschitz var. *purpurea* Making (Scrophulariaceae), *Cornus officinalis* Siebold et Zuccarini (Cornaceae), *Poria cocos* Wolf (Polyporaceae), *Alisma orientale* Juzepczuk (Alismataceae), *Paeonia moutan* Sims (Paeoniaceae), *Lycii cortex Radicis* (Solanaceae), *Scrophularia buergeriana* Miq. (Scrophulariaceae), *Lycium chinense* Miller (Solanaceae), *Rubus coreanum* Miquel (Rosaceae), *Plantago asiatica* L. (Plantaginaceae), *Schizonepeta tenuifolia* Brigue (Labiatae) and *Siler divaricatum* Benth et Hooker (*Ledebouriella seseloides* Wolff), respectively. The extracts of each prescription were prepared by decocting the mixed herbs with 10 times (v/w) of H₂O for 1.5 hrs. After filtration, the residue was boiled for additional 1 hr. Filtrates were mixed together and lyophilized by freezing drier (Labconco,

Preezone) and kept at 4°C. The yield of extracts was 15-20% of a dried ingredient weight.

Treatment of cultured cells

To examine protective effects of herbal extracts on naturally occurring and glutamate-induced cerebrotoneuronal degeneration, cultured cells were treated with several concentrations (0.1 - 1.0 mg/ml) of sterilized herbal extracts. The sterilized extracts were prepared by the following procedures: each extract (1 mg/ml) was dissolved in culture media and sonicated for 10 min by a ultrasonic machine (Bronson, Seattle). The solution was filtered with 0.22 µm filter (Millipore, MA). At the 5th days after beginning the cultures, neural cells were treated with herbal extracts for 3 days and co-treated with glutamate (0.7 - 1.0 mM) at the last day of herbal treatment.

Measurement of LDH release

To verify the damages of cultured cerebrocortical cells, the release of LDH into media was spectrophotometrically assayed by following NADH-oxidation at 340nm (Bergmeyer, 1974). 100ml of media was assayed for the released LDH activity. LDH activity in international unit (IU) of each aliquot was determined by Wroblewski-La Due method using COBAS/FARA II (Roche, Switzerland).

DNA fragmentation analysis

After completion of extract and glutamate treatment, cultured cells were washed with ice-cold PBS and lysed in 2ml of extraction buffer (10mM EDTA, 0.5% sodium dodecyl sulfate, 100 mg/ml proteinase K, 10mM Tris/HCl, pH 8.0) and genomic DNA was extracted as described previously (Heron et al., 1993). DNA samples were analyzed on 1.2% agarose gels containing ethidium bromide (0.5 mg/ml). DNA fragmentation

was visualized by transillumination.

Reagents

Penicillin G, streptomycin, fetal calf serum and Dulbecco's modified eagle's medium containing 2 mM glutamine, 28 mM glucose and 22 mM sodium bicarbonate were obtained from Gibco BRL (Rockville, MD.) LDH assay kit was purchased from Sigma (St. Louis, MO). All other chemicals were of the highest grade from commercial sources.

Statistical analysis

Values obtained in this study are expressed as mean S.E. Statistical comparison was made by Student Newman Keuls procedure to test for significant differences. Difference was considered to be significant at $p < 0.05$

RESULTS

Protection of naturally occurring

cerebroneuronal cell degeneration

Under typical culture conditions, cerebrocortical cells underwent age-dependent neuronal degeneration (data not shown). To clarify the protective effects of SJDBT and SMJHT on naturally occurring neuronal degeneration in cultures, cerebrocortical cells were treated with 1 mg/ml of extracts from 8 to 11 days after beginning the cultures. As shown in Fig. 1, both extracts conspicuously improved the age-dependent degeneration of cerebrocortical cells, especially astrocyte-like cells and its dendrites. These results were also confirmed by measuring LDH activity released into culture media. LDH activity in the SJDBT- and SMJHT (0.1 and 1 mg/ml)-treated cerebrocortical cells was significantly low compared to that in normal cells without extract treatment ($p < 0.05$ or $p < 0.01$, Fig. 2) These results indicate that herbal extracts used here may attenuate and/or protect the naturally occurring cerebroneuronal degeneration.

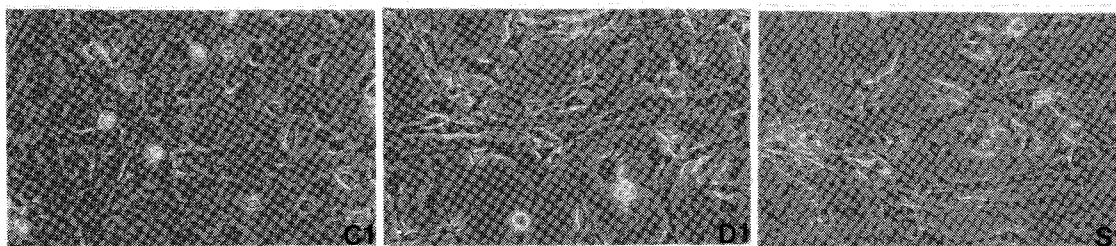


Fig 1. Protection of naturally occurring neuronal degeneration by herbal extracts. Cultured cerebrocortical cells were treated with 1 mg/ml of herbal extracts from 8 to 11 days after beginning cultures, and photographed with a phase-contrast microscope using a green interference filter and Kodak T-Max film, ASA 400. C1, control; D1, Soeumin Sibjeundaibo-tang; S1, Soyangin Sibimijihwang-tang.

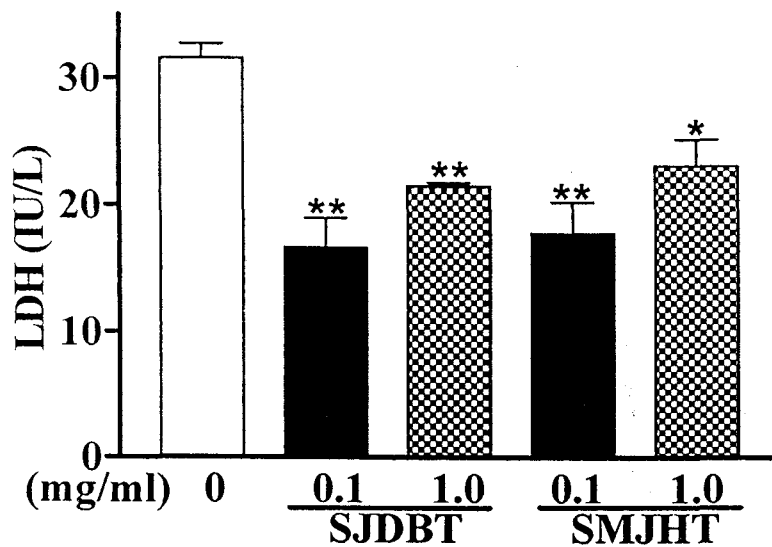


Fig 2. LDH activity released into culture media. Cultured cerebrocortical cells were treated with each concentration of herbal extracts from 8 to 11 days after beginning cultures, and the media at the last day of treatment was collected for analysis. Results are expressed as mean \pm S.E. from 7 experiments. * p <0.05, ** p <0.01 vs. control. SJDBT, Soeumin Sibjeundaibo-tang; SMJHT, Soyangin Sibimijihwang-tang.

Protection of glutamate-induced cerebri-neuronal cell degeneration

Glutamate may play a role in the neuronal degeneration that occurs in Alzheimer's and Huntington's diseases (Maragos *et al.*, 1987; Mattson, 1988). Regarding in these, this study examined the protective effects of SJDBT and SMJHT on glutamate-induced neuronal degeneration in cultured cerebrocortical cells, and results are shown in Figs. 3 and 4. When cerebri-neuronal cells were treated with 1mM of glutamate for 12 hrs at 7 days after beginning the cultures, there was a conspicuous morphological change compared to that in normal cells. In contrast to these, pretreatment of neuronal cells with 0.1 and 1.0 mg/ml of SJDBT (Fig. 3) and SMJHT (Fig. 4) for 2 days obviously attenuated the glutamate-induced neuronal degeneration. These results suggest that SJDBT and SMJHT also protect the glutamate-induced neuronal damages in cultured cerebrocortical cells.

Protection of DNA fragmentation induced by glutamate

To clarify whether apoptotic processes are involved in the protective effects of SJDBT and SMJHT on glutamate-induced cerebri-neuronal degeneration, this study examined the DNA fragmentation in cultured cerebrocortical cells treated with or without herbal extracts in the presence of glutamate. When cells were treated with 0.7 mM of glutamate for 24 hrs at 7 days after beginning the cultures, fragmented DNAs were observed in the electrophoretic analysis. This fragmentation was conspicuously reduced by pretreatment of cerebri-neuronal cells with 1.0 mg/ml of SJDBT and SMJHT (data not shown). These results suggest that a putative apoptotic fragments might be involved in the glutamate-induced cerebri-neuronal degeneration in our system, and this process might be protected by herbal extracts used here.

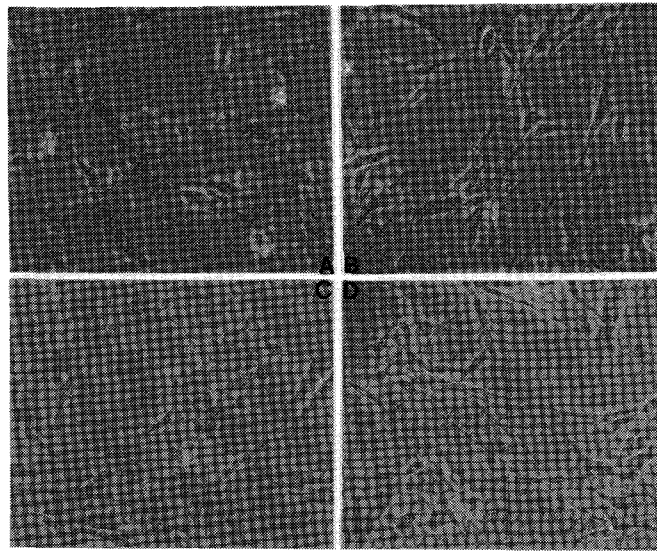


Fig 3. Improvement of glutamate-induced neuronal damages by Soeumin Sibjeundaibo-tang. Cultured cerebrocortical cells were treated with each concentration of herbal extracts from 5 to 7 days after beginning cultures, and 1mM of glutamate was simultaneously added into the media for 12 hrs at the last day of treatment. A, 1 mM glutamate alone; B, 1 mg/ml extracts alone; C, 1 mM glutamate plus 0.1 mg/ml extracts; D, 1 mM glutamate plus 1 mg/ml extracts.

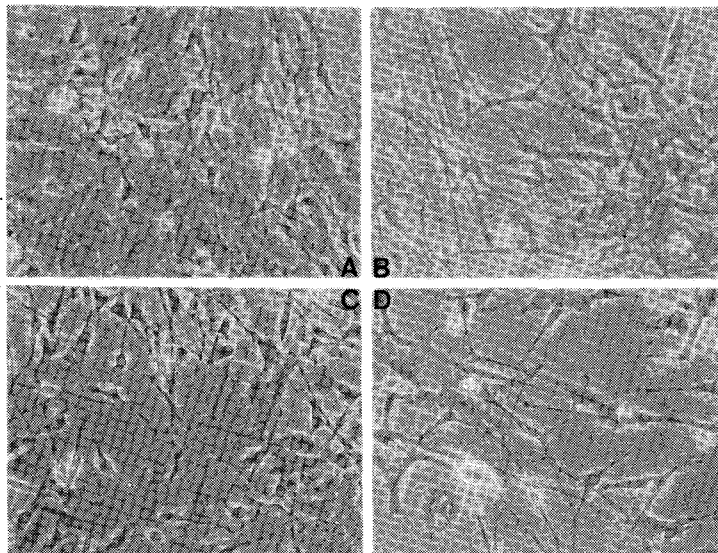


Fig 4. Improvement of glutamate-induced neuronal damages by Soyangin Sibimijihwang-tang. Experimental procedure was the same one as described in Fig. 3. A, 1 mM glutamate alone, B, 1 mg/ml extracts alone; C, 1 mM glutamate plus 0.1 mg/ml extracts; D, 1 mM glutamate plus 1 mg/ml extracts.

DISCUSSION

In general, the prescriptions of oriental medicine are often prepared with the combination of several natural products as empirical knowledge and expected show be more potent pharmacological activity compared to using a single component in the treatment of variety of diseases. It may be important to verify the pharmacological properties of prescription of oriental medicine using the entire extracts, because this gives us a useful information to improve their pharmacological properties in the scientific regards. Based on these, this study using the entire extracts of prescription suggests that SJDBT and SMJHT may protect and/or attenuate the naturally occurring and excitotoxicity-induced neuronal degeneration in the cultured mice cerebrocortical cells.

Although there has been widespread speculation that apoptosis is the cause of neuronal degeneration in many, if not all, neurodegenerative diseases (Lane *et al.*, 1996), the mechanism underlying the delayed neuronal cell death that leads to DNA fragmentation following neuronal damage remains to be determined. This is due to the difficulty of acquiring affected human tissue near the time of death and the paucity of good animal models for neurodegenerative diseases. Much of our understanding of the biochemical and molecular events associated with neuronal apoptosis has come from the study of cultured central and peripheral neurons as well as work from cell lines that exhibit neuronal properties. Using cultured neuronal cells, we observed that matured cerebrocortical neuronal cells underwent an age-dependent degeneration under a typical culture condition, and these results were well correlated with the previous reports (Dessi *et al.*, 1995; Ishitani *et al.*, 1996b). Although we could not decisively suggest whether apoptotic cell death was involved in the age-induced neuronal degeneration in our

system, it might be importantly related to the neuronal degeneration of cultured cerebrocortical cells, because apoptotic neuronal death is an active process in which cells "commit suicide" by mechanisms that depend on de novo synthesis of "killer protein" (Johnson and Deckwerth, 1993; Morris and Geller, 1996). If this hypothesis is true, cerebrouneuron degeneration observed here might be caused by both apoptotic and necrotic processes, because apoptotic cells can eventually undergo secondary necrosis and rupture their contents into the surrounding medium (Bonfoco *et al.*, 1995). This may be also supported by the results described in the Fig. 2. When cultured cerebrocortical cells were treated with SJDBT and SMJHT, age-induced cerebrocortical degeneration was conspicuously attenuated in morphological observation, and this was consistent with the LDH activity released into surrounding culture media, indicating that LDH activity was significantly decreased by treatment of cerebrouneuron cells with SJDBT and SMJHT. These results might suggest that naturally occurring cerebrocortical degeneration observed in this study might be resulted from the apoptotic and necrotic processes, and cellular damages by these might be protected by herbal extracts tested here. However, it remains to be clarified whether the primary target for their pharmacological activities is involved in the protection of age-dependent apoptotic cerebrouneuron death.

By observing morphological changes and DNA fragmentation, we investigated the protective effects of herbal extracts on the glutamate-induced neuronal damages in cultured cerebrocortical cells. Exposure to high concentration of glutamate resulted in rapid mitochondrial swelling and disruption of mitochondrial matrices, and this early pattern of massive mitochondrial injury is typical of necrosis *in vivo* (Wyllie *et al.*, 1980) and can not be ameliorated by superoxide dismutase and catalase (Bonfoco *et al.*, 1995).

We observed that double fluorescence staining with ethidium bromide and acridine orange revealed numerous necrotic cerebri neuronal cells treated with glutamate (1mM) at 7 days after beginning the cultures (data not shown). These results suggested that glutamate-induced cerebrocortical degeneration might be also mediated, in part, by a necrotic neuronal damage, and these were conspicuously attenuated by pretreatment of SJDBT and SMJHT. Although the biochemical mechanisms involved in these effects could not be described here, the pharmacological effects of SJDBT and SMJHT which protect the age-dependent and glutamate-induced neuronal damages might be meaningful evidences for prevention and attenuation of aged brain with a neurodegenerative diseases. In addition, the previous reports suggested that immature neuronal cells in cortical cultures were apparently resistant to the glutamate-induced neuronal toxicity, and the basis of for the resistance of immature neurons to excitotoxin was explained by reasoning that the immature neurons contain fewer membrane receptors for glutamate than the mature neurons (Koh and Choi, 1987; Lustig *et al.*, 1992). In our experiments, DNA fragmentation was detected in the cultured cerebri neuronal cells treated with relatively high concentration (0.7mM) of glutamate at 7 days after beginning the cultures, and these results could be also supported by morphological evidences demonstrated by double fluorescence stain (data not shown). Therefore, results obtained here lead us to consider that the cerebri neuronal damages might be mediated by a apoptotic and necrotic pathways, and these pathological processes might be protected by SJDBT and SMJHT, resulted in improvement of neuronal viability and DNA fragmentation.

In conclusion, this study suggests that one of pharmacological properties of SJDBT and SMJHT is the attenuation and protection of naturally occurring and glutamate-induced

cerebri neuronal degeneration in culture. These prescriptions may be considered as one of useful medicines that can prevent and/or treat the cerebri neuronal impairment resulted from age-dependent and excitotoxicity-induced neuronal degeneration in human brain.

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