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# Protective effect of *Shenqi-wan* and its fractions on *N*-methyl-D-aspartate-induced excitotoxicity in rat hippocampus

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**SUMMARY**

*Shenqi-wan*, Oriental herbal medicine formulation, has traditionally been used for the treatment of delayed mental and physical development in children, complications of diabetes, and glomerulonephritis. In the present study, we investigated the protective effect of the aqueous extract of *Shenqi-wan* and its fractions against *N*-methyl-D-aspartate (NMDA)-induced exitotoxicity in rat hippocampal CA1 neurons. Fractions were elucidated at 0 - 10 min, 11 - 20 min, and 21 - 30 min by using gravity column chromatography method. In the present results, treatment with NMDA on cultured hippocampal slices induced neuronal death in the hippocampal CA1 region. Pre-treatment with the *Shenqi-wan* did not exerted protective effect, however its fractions suppressed NMDA-induced neuronal damage. The fraction elucidated at 11 - 20 min showed the most potent protective effect. These results revealed that effective substances of the *Shenqi-wan* against NMDA-induced excitotoxicity may exist mainly in the fraction elucidated at 11 - 20 min.

**Key words:** *Shenqi-wan*; Fractions; *N*-methyl-D-aspartate; Hippocampus; CA1 region

## ITRODUCTION

Glutamate is major excitatory neurotransmitter and it has two types of receptors in the mammalian brain. One is ionotropic receptors divided *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methylisoxazole-3-propionate (AMPA) receptors, and kainate (KA) receptors. They open glutamate-gated cation channels, and are subdivided into NMDA and non-NMDA by $Ca^{2+}$ permeability. Another is metabotropic receptors (mGluRs), as G-protein coupled receptors (Verkhratsky and Kirchhoff, 2007).

Ionotropic glutamate recepters play a crucial role in the pathology of cerebral ischemia and stroke (Andras *et al.*, 2007). Because of decreased cerebral blood flow in ischemia and stroke, induces reduction of oxygen, finally ATP depletion and increase of extracellular glutamate. In this situation, ionotropic glutamate receptors induce excessive intracellular $Ca^{2+}$ release that initiates several metabolic pathways, and leads to neuronal cell death, that is called 'excitotoxicity'. To reduce excitotoxicity, it was shown that ionotropic glutamate receptors antagonists exerted protective effect on neuronal cell death and also reduced brain edema induced by cerebral ischemia and

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intracerebral hemorrhage (Andras *et al.,* 2007).

*Shenqi-wan*, an Oriental herbal formulation, is traditionally been used for the treatment of delayed mental and physical development in children, complications of diabetes, and glomerulonephritis patients (Wang and Zhang, 1987; Chen *et al.,* 1997). However, the effect of *Shenqi-wan* on NMDA-induced excitotoxicity has not been reported yet. In the present study, the protective effects of the aqueous extract of *Shenqi-wan* and its fractions on NMDA-induced excitotoxicity were investigated using by organotypic rat hippocampal slice culture.

## MATERIAL AND METHODS

### Preparation of the aqueous extract of *Shenqi-wan* and its fractions

The ingredients of *Shenqi-wan* are as follows: *Rehmanniae Radix* 16 g, *Dioscorae Radix* 8 g, *Corni Fructus* 8 g, *Alimatis Rhizoma* 6 g, *Moutan Cortex Radicis* 6 g, *Hoelen* 6 g, *Maximowicziae Fructus* 8 g, and *Cervi Cornu* 4 g. All ingredients were obtained from the Kyung Dong marketplace (Seoul, Republic of Korea). After washing, to obtain the aqueous extracts of *Shenqi-wan*, the ingredients were added to distilled water, heat-extracted, pressure-filtered, concentrated with rotary evaporator, and lyophilized (EYELA, Tokyo, Japan). The resulting powder, weighing 15.48 g (a yield of 24.97%) was diluted to the concentrations needed with distilled water and filtered through a 0.22 μm syringe filter before use.

To obtain the fractions of lyophilized aqueous extraction of *Shenqi-wan*, gravity column chromatography method was used. In briefly, 5 g of lyophilized aqueous extraction of *Shenqi-wan* was dissolved with 5 ml distilled water and loaded to 5 cm diameter glass column filled up about three forth with Diaion® HP-20, mobile phase was distilled water. The fractions were collected at every 30 s used by Retriever® 500, and terminated at 30 min after. The fractions were merged at 0 to 10 min (fraction 1), 11 to 20 min (fraction 2), and 21 to 30 min (fraction 3), and they were concentrated with rotary evaporator and lyophilized (EYELA, Tokyo, Japan). The resulting powders weighing 2.05 g (a yield of 41%) in fraction1, 1.20 g (a yield of 24%) in fraction 2, and 0.292 g (a yield of 5.84% ) in fraction 3 were diluted to the concentrations needed using distilled water, and filtered through a 0.22 μm syringe filter before use.

### Organotypic slice culture

Organotypic hippocampal slice culture was prepared by a previously described method (Lee *et al.,* 2003). The hippocampi of Sprague-Dawley rats (postnatal day 7) were isolated and cut transversely at a thickness of 350 μm using a McILWAIN tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). The slices were placed on Millicell-CM inserts (Millipore) in 6 well plates that contained 1 ml of culturing medium composed of 50% minimum essential media α-modification (α-MEM), 25% Hank's balanced salts solution (HBSS) and 25% horse serum. The slices were cultured for 14 days at 36°C in a 5% $CO_2$ incubator, and the medium was changed every third day. This experiment was designed to investigate the protective effect of aqueous extraction of *Shenqi-wan* and its fractions on NMDA-induced hippocampal neuronal damage. The slice cultures were divided into 10 groups: the control group, the $10^{-4}$ M NMDA-treated group, the 0.1 mg/ml *Shenqi-wan* pre-treated and $10^{-4}$ M NMDA-treated group, the 1 mg/ml *Shenqi-wan* pre-treated and $10^{-4}$ M NMDA-treated group, the 0.1 mg/ml fraction 1 pre-treated and $10^{-4}$ M NMDA-treated group, the 1 mg/ml 0.1 mg/ml fraction 1 pre-treated and $10^{-4}$ M NMDA-treated group, the 0.1 mg/ml fraction 2 pre-treated and $10^{-4}$ M NMDA-treated group, the 1 mg/ml fraction 2 pre-treated and $10^{-4}$ M NMDA-treated group, the 0.1 mg/ml fraction 3 pre-treated and $10^{-4}$ M NMDA-treated group, and the 1 mg/ml fraction 3 pre-treated and $10^{-4}$ M NMDA-treated group. NMDA was treated for 48 h and aqueous extracts of *Shenqi-wan* and its fractions were pre-treated 1 h before the NMDA exposure in hippocampal slice cultures.

Propidium iodide (PI, 5 mg/ml) was added to each well and PI stained images were captured under same exposure using an inverted fluorescence microscope with an attached digital CCD camera (Axiovert S100, Zeiss, Göttingen, Germany). The observed areas were measured using the ImagePro® analysis software (version 1.52), and the percentage of neuronal death was then calculated.

**Drugs**

α-MEM and HBSS used in this experiment were obtained from JBI (Daegu, Republic of Korea) and all other drugs used in this experiment were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

**Statistics**

The results were presented as the mean ± standard error of the mean (S.E.M.). Statistical analysis was made by one-way ANOVA followed by Duncan *post-hoc* test. The differences were considered significantly at $P < 0.05$.

## RESULTS

**Effect of aqueous extract of *Shenqi-wan* on NMDA-induced neuronal damage**

NMDA-induced neuronal damage in the pyramidal layer of the hippocampal CA1 region was visualized by PI staining, which is preferentially taken up into nonviable cells. In the control group, 4.1 ± 1.6% of PI staining was observed. After 48 h of exposure to NMDA, the level of PI uptake was markedly increased, and most of the cells in the pyramidal layer of the hippocampal CA1 were stained with PI. PI uptake in the groups pre-treated with the aqueous extract of *Shenqi-wan* at the concentrations of 0.1 mg/ml and 1 mg/ml was 90.7 ± 8.6% and 82.1 ± 15.9%, respectively. The present results showed that the aqueous extract of *Shenqi-wan* exerted no significant effect on NMDA-induced neuronal cell death in rat hippocampal CA1 region (Fig. 1).

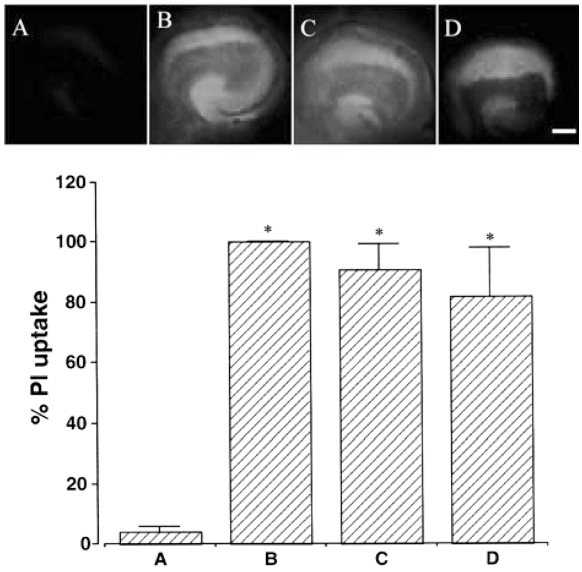


**Fig. 1.** Effect of *Shenqi-wan* on PI uptake in hippocampal slice culture. Upper: Photomicrographs of PI-assayed culture. The scale bar represents 400 μm. Lower: Level of PI staining in each group. (A) Control group, (B) $10^{-4}$ M NMDA-treated group, (C) 0.1 mg/ml *Shenqi-wan* pre-treated and $10^{-4}$ M NMDA-treated group, and (D) 1 mg/ml *Shenqi-wan* pre-treated and $10^{-4}$ M NMDA-treated group. The results are presented as mean ± S.E.M. *represents $P < 0.05$ compared to the control group.

**Effect of the fraction 1 of *Shenqi-wan* on NMDA-induced neuronal damage**

NMDA-induced neuronal damage in the pyramidal layer of the hippocampal CA1 region was visualized by PI staining, which is preferentially taken up into nonviable cells. In the control group, 4.1 ± 1.6% of PI staining was observed. After 48 h of exposure to NMDA, the level of PI uptake was markedly increased, and most of the cells in the pyramidal layer of the hippocampal CA1 were stained with PI. PI uptake in the groups pre-treated with fraction 1 of *Shenqi-wan* at the concentrations of 0.1 mg/ml and 1 mg/ml was 94.2 ± 6.2% and 94.8 ± 8.6%, respectively. The present results showed that the fraction 1 of *Shenqi-wan* exerted no significant effect on NMDA-induced neuronal cell death in rat hippocampal CA1 region (Fig. 2).

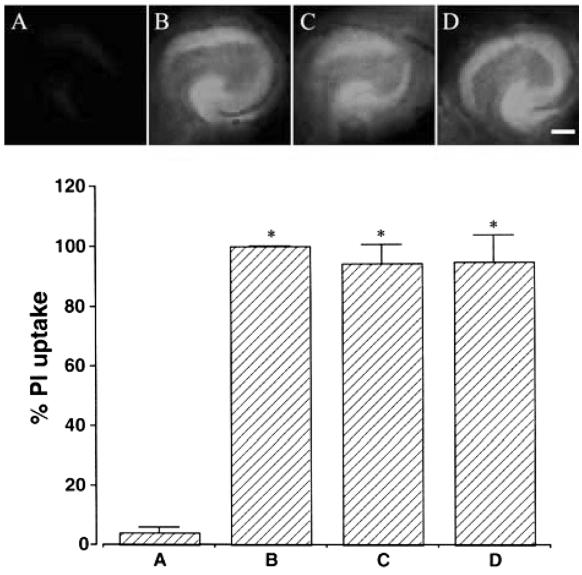


**Fig. 2.** Effect of fraction 1 of *Shenqi-wan* elucidated at 0-10 min on PI uptake in hippocampal slice culture. Upper: Photomicrographs of PI-assayed culture. The scale bar represents 400 μm. Lower: Level of PI staining in each group. (A) Control group, (B) $10^{-4}$ M NMDA-treated group, (C) 0.1 mg/ml fraction 1 pre-treated and $10^{-4}$ M NMDA-treated group, and (D) 1 mg/ml fraction 1 and $10^{-4}$ M NMDA-treated group. The results are presented as mean ± S.E.M. *represents $P < 0.05$ compared to the control group.

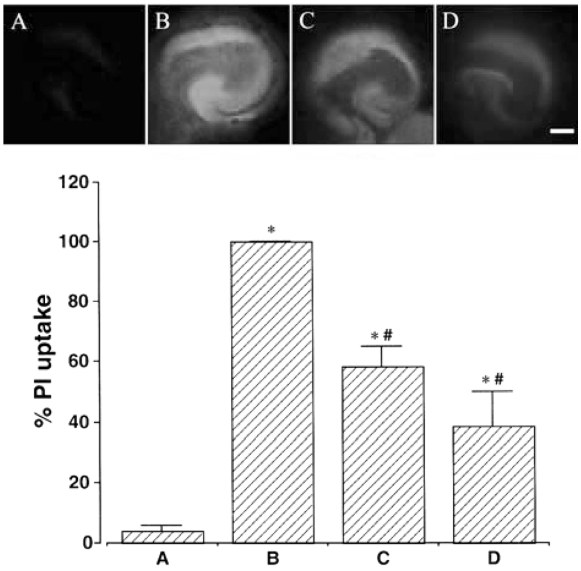


**Fig. 3.** Effect of fraction 2 of *Shenqi-wan* elucidated at 11 - 20 min on PI uptake in hippocampal slice culture. Upper: Photomicrographs of PI-assayed culture. The scale bar represents 400 μm. Lower: Level of PI staining in each group. (A) Control group, (B) $10^{-4}$ M NMDA-treated group, (C) 0.1 mg/ml fraction 2 pre-treated and $10^{-4}$ M NMDA-treated group, and (D) 1 mg/ml fraction 2 and $10^{-4}$ M NMDA-treated group. The results are presented as mean ± S.E.M. *represents $P < 0.05$ compared to the control group. #represents $P < 0.05$ compared to the NMDA-treated group.

**Effect of fraction 2 of *Shenqi-wan* on NMDA-induced neuronal damage**

NMDA-induced neuronal damage in the pyramidal layer of the hippocampal CA1 region was visualized by PI staining, which is preferentially taken up into nonviable cells. In the control group, 4.1 ± 1.6% of PI staining was observed. After 48 h of exposure to NMDA, the level of PI uptake was markedly increased, and most of the cells in the pyramidal layer of the hippocampal CA1 were stained with PI. PI uptake in the groups pre-treated with fraction 2 of *Shenqi-wan* at the concentrations of 0.1 mg/ml and 1 mg/ml was 58.4 ± 6.7% and 38.4 ± 11.6%, respectively. The present results showed that fraction 2 of *Shenqi-wan* exerted protective effect on NMDA-induced neuronal cell death in rat hippocampal CA1 region as dose-dependant manner (Fig. 3).

**Effect of fraction 3 of *Shenqi-wan* on NMDA-induced neuronal damage**

NMDA-induced neuronal damage in the pyramidal layer of the hippocampal CA1 region was visualized by PI staining, which is preferentially taken up into nonviable cells. In the control group, 4.1 ± 1.6% of PI staining was observed. After 48 h of exposure to NMDA, the level of PI uptake was markedly increased, and most of the cells in the pyramidal layer of the hippocampal CA1 were stained with PI. PI uptake in the groups pre-treated with fraction 3 of *Shenqi-* at the concentrations of 0.1 mg/ml and 1 mg/ml was 88.4 ± 6.1% and 55.8 ± 12.8%, respectively. The present results showed that 1 mg/ml fraction 3 of *Shenqi-wan* exerted protective effect on NMDA-induced neuronal cell death in rat hippocampal CA1 region (Fig. 4).

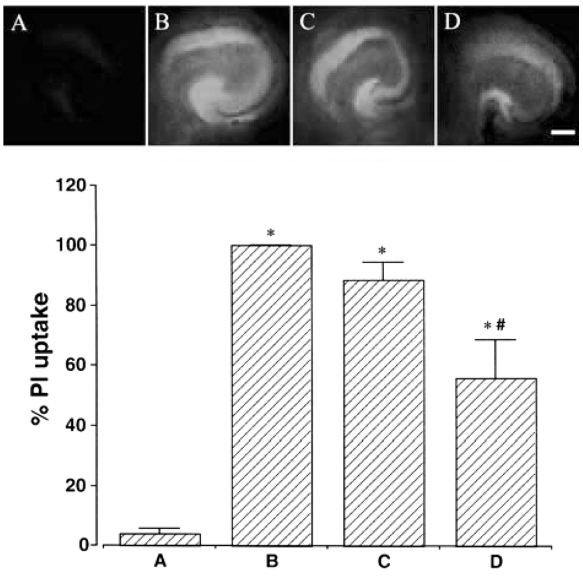


**Fig. 4.** Effect of fraction 3 of *Shenqi-wan* elucidated at 21 - 30 min on PI uptake in hippocampal slice culture. Upper: Photomicrographs of PI-assayed culture. The scale bar represents 400 μm. Lower: Level of PI staining in each group. (A) Control group, (B) $10^{-4}$ M NMDA-treated group, (C) 0.1 mg/ml fraction 3 pre-treated and $10^{-4}$ M NMDA-treated group, and (D) 1 mg/ml fraction 3 pre-treated and $10^{-4}$ M NMDA-treated group. The results are presented as mean ± S.E.M. $^{*}$represents $P < 0.05$ compared to the control group. $^{\#}$represents $P < 0.05$ compared to the NMDA-treated group.

## DISCUSSION

In the present study, we compared the effects of the aqueous extract of *Shenqi-wan* and its fractions on NMDA-induced excitotoxicity in rat hippocampal CA1 region. In the present results, excitotoxicity induced by glutamate has been documented. It was reported that AMPA and KA receptors mediate ischemic damage in white matter axon (McCarran and Goldberg, 2007), and $Ca^{2+}$ permeable AMPA channel contributed sporadic amyotrophic lateral sclerosis, Alzhemer's disease, and epilepsy (Kwak and Weiss, 2006). However, NMDA receptors and its effects are mainly targeted in exitotoxicity among the ionotropic glutamate subtype receptors. It is well known that hippocampal CA1 region is more vulnerable to excitotoxicity compared to CA3 region, because of different existence of NMDA receptors response (Gee *et al.*, 2006).

Increased extracellular concentration of glutamate overstimulates NMDA receptors resulting in increased $Ca^{2+}$ influx, which in turn disables mitochondrial functions (Tong *et al.*, 1995), rapidly increases the concentration of cytoplasmic reactive oxygen species (Gunasekar *et al.*, 1995), and ultimately causes neuronal cell death. Because NMDA receptors play a crucial role in glutamate-induced acute neuronal damage, NMDA receptor antagonists are thought to reduce neuronal cell death during and following ischemic attacks (Simon *et al.*, 1984).

Kim *et al.* (2007) showed the activation of protein kinase C through cyclooxygnase-2 (COX-2) pathway inducted neuroprotective effect on NMDA-induced ischemia, and Xu *et al.* (2007) suggested that NMDA receptors activated prosuvival pathway.

In the present results, the aqueous extract of *Shenqi-wan* at concentrations of 0.1 mg/ml and 1 mg/ml did not show significant protective effect on NMDA-induced exitotoxicity in rat hippocampal CA1 neurons. Shin *et al.* (2003) reported that *Shenqi-wan* has neuroprotective effect on $H_2O_2$-induced damage in HiB5 cell line and they also showed that this neuroprotective effect of *Shenqi-wan* on exitotoxicity was induced by suppressing glutamate-activated and NMDA-activated ion currents in rat hippocampal CA1 neurons. Also Yang *et al.* (2006) reported that *Liuweidihuang* decoction, similar to *Shenqi-wan,* suppressed $K^{+}$ and $Ca^{2+}$ ion currents in cultured rat hippocampal neurons.

In the present study, we confirmed that the concentrations of the aqueous extract of *Shenqi-wan* used in this study exerted no significant neuroprotective effect on NMDA-induced excitotoxicity in rat hippocampal CA1 neurons. The fraction elucidated during 0 - 10 min also showed no significant protective effect. The

fractions, however, elucidated during 11 - 20 min and during 21 - 30 min showed neuroprotective effect on NMDA-induced excitotoxicity. The fraction elucidated during 11 - 20 min exerted most potent protective effect.

Here in this study, we suggest that the active substances for the neuroprotection of the aqueous extract of *Shenqi-wan* on NMDA-induced excitotoxicity in rat hippocampal CA1 neurons mainly exist in the fraction elucidated during 11 - 20 min. Additional studies on the effect of each ingredient herbs of *Shenqi-wan* and on the mechanism of this herbal formulation may yield novel ideas with possible implications for further therapeutic approaches.

## ACKNOWLEDGEMENTS

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