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

Joung-Hun Lee¹, Youn-Sub Kim¹, Young-Sick Kim², Sung-Eun Kim³, Yun-Hee Sung³, Bo-Kyun Kim³, Jin-Woo Lee⁵, Dae-Hyun Ham³, Hyejung Lee³ and Chang-Ju Kim¹,²,³,*

¹Department of Anatomy-Pointology, College of Oriental Medicine, Kyungwon University, 65 Bokjung-dong, Sujung-gu, Songnam 461-701, Republic of Korea; ²Department of Physiology, College of Medicine, Kyung Hee University; ³Acupuncture and Meridian Science Research Center, Kyung Hee University, 1 Hoigi-dong, Dongdaemoon-gu, Seoul 130-701, Republic of Korea

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 excitotoxicity 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

INTRODUCTION

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 Ca²⁺ permeability. Another is metabotropic receptors (mGluRs), as G-protein coupled receptors (Verkhratsky and Kirchhoff, 2007).

Ionotropic glutamate receptors 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 Ca²⁺ 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
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, _Dioscoreae Radix_ 8 g, _Corni Fructus_ 8 g, _Alpinis 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 powders weighing 2.05 g (a yield of 41%) in fraction 1, 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.

To obtain the fractions of lyophilized aqueous extraction 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 powders 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.

_Aquaporin 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% CO₂ 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 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).

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

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-wan 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).
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, Alzheimer’s disease, and epilepsy (Kwak and Weiss, 2006). However, NMDA receptors and its effects are mainly targeted in excitotoxicity 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 cyclooxygenase-2 (COX-2) pathway induced 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 excitotoxicity in rat hippocampal CA1 neurons. Shin et al. (2003) reported that Shenqi-wan has neuroprotective effect on H\(_2\)O\(_2\)-induced damage in HiB5 cell line and they also showed that this neuroprotective effect of Shenqi-wan on excitotoxicity 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**

This work was supported by the SRC/ERC program of MOST/KOSEF (R11-2005-014).

**REFERENCES**


