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PROTECTIVE EFFECT OF METHANOL EXTRACT ZIZYPHI SPINOSI SEMEN (ZSS) ON EXCITATORY AMINO ACIDS-INDUCED NEUROTOXICITY IN CULTURED RAT CEREBELLAR GRANULE NEURON

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Introduction

Glutamate is the main excitatory neurotransmitter (EAA) in the mammalian brain and mediates fast excitatory transmission via three classes of ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) and kainite. Glutamate excitotoxicity plays a significant role in the pathogenesis of several acute and chronic neurological condition. Substantive evidence supports the involvement of glutamate in neuronal death or injury associated with ischemic brain injury, stroke, status epilepticus, amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease.[1] Over stimulation of NMDA receptor with glutamate results in an excessive influx of Ca^{2+} that culminates in the activation of a plethora of potentially neurotoxic mechanisms, such as kinases, proteases, phospholipases and free radical production. Antagonism of glutamatergic receptors with competitive or non-competitive antagonists, such as MK-801, AP-5 or DNQX, protects neurons against EAA-mediated cell death, both in cell cultures and in cerebral ischemia models.

A medical plant named Zizyphi Spinosi Semen (ZSS) can calm the mind, reserve *qi*, nourish muscle, and enrich bone marrow. In recent laboratory investigations, the herb showed a tranquilzing and hypnotic effect. It can significantly increase sleep time induced by hexobarbital. It is also an analgesic and anticonvulsive agent. The present study evaluated the effects of crude methanol extracts prepared from ZSS on EAA-induced neurotoxicity and characterized its action mechanism in primarily cultured cerebellar granule neurons, an in vitro well characterized system for studying neuronal viability.

Methods and Materials

Primary cultures of rat cerebellar neurons were prepared from 8-day-old Sprague-Dawley rat pups. After being dissociated by trituration and tryptinization, cells were seeded on poly-lysine coated multiwell plate (2×10^6/ml). Cells were treated with cytosine arabinoside to eliminate glial cells 48

hours after the seeding. Mature cells of 7-10 days in vitro were used for experiments. For experiments, cells were washed to remove medium and placed in a HEPES-buffered incubation buffer containing 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 5.6 mM glucose and 8.6 mM HEPES (pH 7.4). Cells were incubated with EAAs for 3 to 6 hrs in the presence or absence of ZSS or other inhibitors. Trypan blue exclusion test were performed to measure neuronal cell death induced by EAAs.[2] The amount of glutamate secreted into the buffer was quantified by HPLC with an electrochemical detector (ECD) after precolumn derivatization of sample aliquots with O-phthaldialdehyde/2-mercaptoethanol (OPA/2-ME).[3] Intracellular Ca^{2+}concentration ($[Ca^{2+}]_i$) was determined with ratio fluorometry using fluorescence dye, fura-2 AM.[4] Intracellular reactive oxygen species (ROS) levels were measured by confocal microscopy (Bio-rad, MRC1021ES) using the dye 2',7'-dichlorofluorescin diacetate (DCF-DA).

Results and Discussion

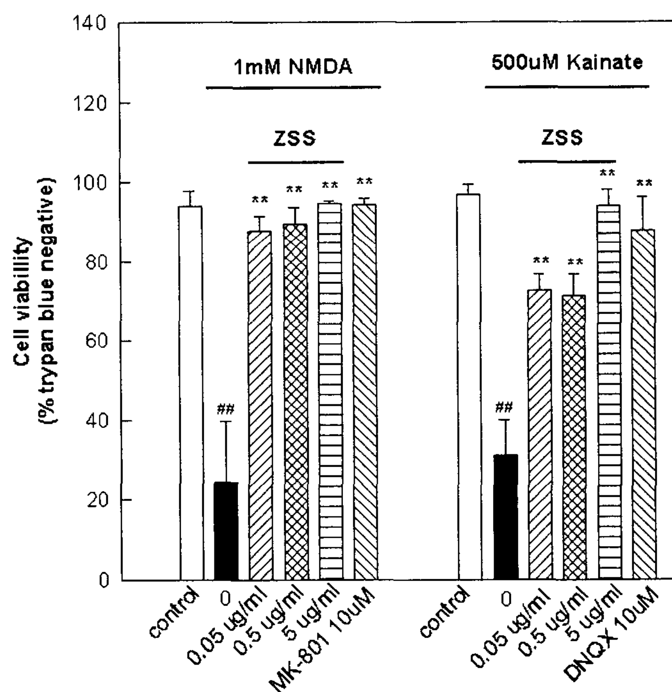

Fig. 1. Effects of ZSS (0.05, 0.5, 5μg/ml), MK-801 (10μM) and DNQX (10μM) on 1mM NMDA and 500μM kainate-induced cell death in cerebellar granule neuron. Cells were incubated with NMDA and kainate for 6hr and 3hr, respectively, in the presence or absence of inhibitors. Inhibitors including ZSS were applied 15 min prior to EAAs. Value represent mean±SEM. ## $p<0.01$ compared to control. ** $p<0.01$ compared to 1mM NMDA or 500μM kainate.

Exposure of primary cultures of cerebellar granule cells for 6hrs to 1mM NMDA or for 3hrs to 500μM kainate resulted in cell death as it was evidenced by Trypan blue exclusion test (Fig. 1). The morphological changes of cerebellar granule neurons after exposure to EAAs were characterized by decomposition of neuronal aggregates, fragmentation of neurite and loss of soma. Fig. 1 shows that NMDA and kainate-induced neuronal cell death was attenuated by MK-801 (the selective non-competitive NMDA receptor antagonist) and DNQX (the non-NMDA receptor antagonists), respectively. ZSS exhibited protective action against NMDA and kainate-induced excitotoxicity in concentration of 0.05, 0.5 and 5 μg/ml. As shown in Fig. 2, 1mM NMDA and 500μM kainate markedly elevated extracellular glutamate level to 1.14 μM, 1.89 μM from 0.43 μM of control, respectively. ZSS strongly blocked the NMDA and kainate-induced elevation of glutamate release showing 0.59 μM, 0.88 μM at the concentration of 0.5 μg/ml, respectively. MK-801 (10μM) and DNQX (10 μM) also significantly inhibited the elevation of glutamate release.

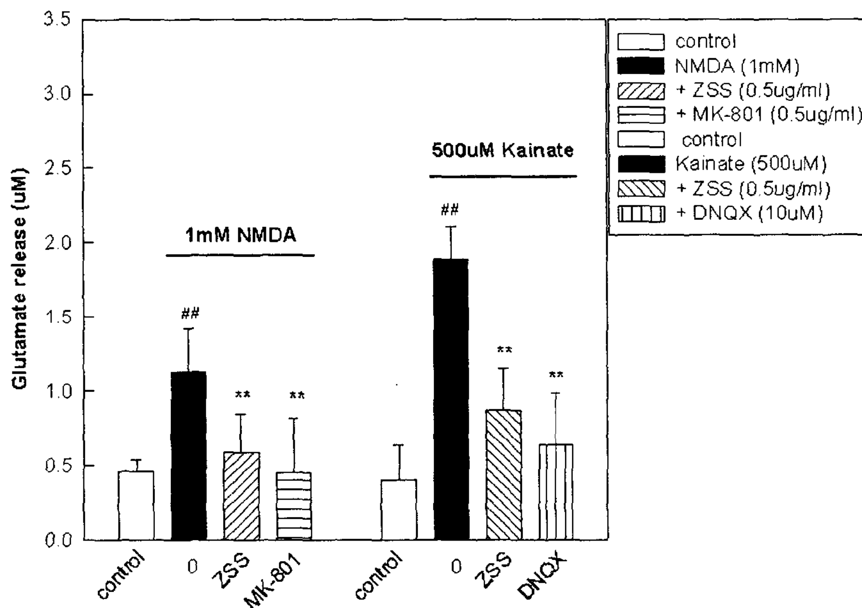

Fig. 2. Inhibitory effects of ZSS, MK-801 and DNQX on NMDA and kainite-induced glutamate release into medium in cerebellar granule neurons. Cells were incubated with NMDA and kainate for 1hr in the presence of absence of inhibitors. Inhibitors including ZSS were applied 15 min prior to EAAs. At the end of the incubation, released glutamate was measured by HPLC with ECD. Value represent mean±SEM. ## $p<0.01$ compared to control. ** $p<0.01$ compared to 1mM NMDA or 500μM kainate.

Increase of $[Ca^{2+}]_i$ has been postulated to be associated with cell death in many studies. The fluorescence intensity ratio of 340 nm excitation to 380 nm excitation (F340/F380) from Fura-2 AM

loaded cell is proportional to $[Ca^{2+}]_i$. As shown in Fig. 3., $[Ca^{2+}]_i$ was gradually increased by 1mM NMDA and 50μM kainate treatment. NMDA and kainate-induced $[Ca^{2+}]_i$ elevation was also completely blocked by 10μM MK-801 and 10μM DNQX, respectively. ZSS completely blocked the NMDA-induced $[Ca^{2+}]_i$ elevation and partially blocked the kainate-induced $[Ca^{2+}]_i$ elevation. In addition, Exposure of DCF-loaded cerebellar granule cells to 1 mM NMDA or 500 μM kainate resulted in an increase in DCF fluorescence, which indicates an increase in the intracellular concentration of ROS. Meanwhile, when neurons were pre-loaded with ZSS, MK-801 and DNQX, the mean fluorescence increase was markedly reduced.

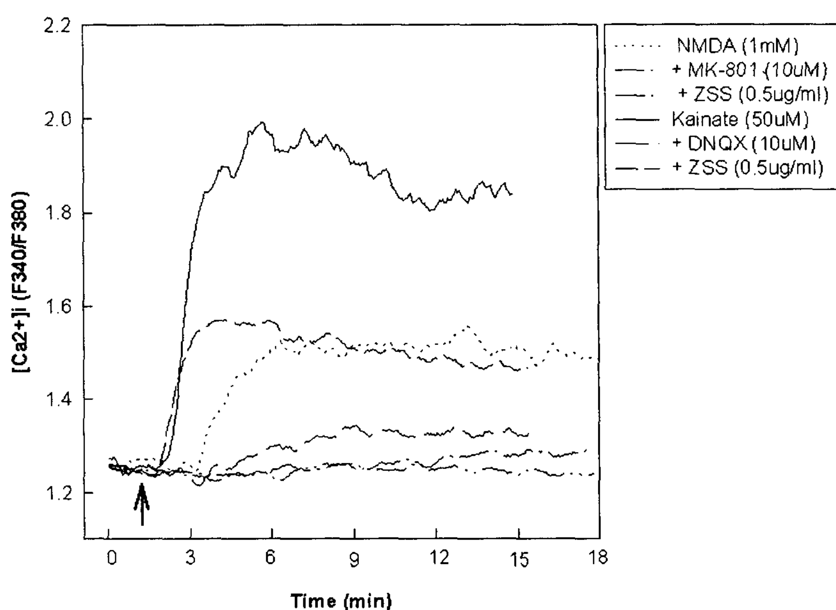

Fig. 3. Change of $[Ca^{2+}]_i$ in response to NMDA and kainate in the presence or absence of ZSS, MK-801 and DNQX in cerebellar granule neurons. Change of $[Ca^{2+}]_i$ was measured with ratio fluorometry using fluorescent dye, fura-2 AM. ZSS, MK-801 and DNQX were applied 15 min pior to NMDA and kainate. $[Ca^{2+}]_i$ was monitored using ratio fluorometry. In the plots shown, each line represents F340/F380 ratio from a representative cell population.

These results demonstrated that ZSS methanol extract exhibited protective action against excitotoxic neuronal death, and the neuroprotective action was related to a series of cell responses consisting of glutamate release and oxidant generation following $[Ca^{2+}]_i$ elevation via glutamate receptors (NMDA and kainite receptors), and resultant cell death, and that ZSS inhibited the NMDA and kainite-induced cell death by the blockade of the NMDA and kainite-induced Ca^{2+} influx. However, the precise mechanism should be defined. It is hoped that these extracts might be of value in preventing neurodegenerative pathophysiological condition.

References

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