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Crude Extract of Zizyphi Jujube Semen Protects Kainic Acid-induced Excitotoxicity in Cultured Rat Neuronal Cells

Jeong Hee Park, Ju Yeon Ban, Hyun Soo Joo, Kyung Sik Song[1], Ki Whan Bae[2] and Yeon Hee Seong*

College of Veterinary Medicine and Research Institute of Veterinary Medicine,
Chungbuk National University, Cheongju, Chungbuk 361-763, Korea
[1]*College of Agriculture and Life-Sciences, Kyungpook National University, Daegu 702-701, Korea*
[2]*College of Pharmacy, Chungnam National University, Taejon 305-764, Korea*

Abstract – Zizypus is one of the herbs widely used in Korea and China due to the CNS calming effect. The present study aims to investigate the effect of the methanol extract of Zizyphi Jujube Semen (ZJS) on kainic acid (KA)-induced neurotoxicity in cultured rat cerebellar granule neuron. ZJS, over a concentration range of 0.05 to 5 μg/ml, inhibited KA (500 μM)-induced neuronal cell death, which was measured by a trypan blue exclusion test and a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Pretreatment of ZJS (0.5 μg/ml) inhibited KA (50 μM)-induced elevation of cytosolic calcium concentration ($[Ca^{2+}]_c$), which was measured by a fluorescent dye, Fura 2-AM, and generation of reactive oxygen species (ROS). ZJS (0.5 μg/ml) inhibited glutamate release into medium induced by KA (500 μM), which was measured by HPLC. These results suggest that ZJS prevents KA-induced neuronal cell damage in vitro.

Keywords – Zizyphi Jujube Semen, Kainic acid, Neurotoxicity, Cerebellar granule cells

Introduction

Glutamate is the major excitatory transmitter as well as an important neurotoxin in the CNS. Elevated extracellular glutamate levels have been shown to affect neuronal activity profoundly by activating specific ionotropic and metabotropic receptors and have been implicated in neurodegenerative processes associated with ischemia and other neuropathological conditions (Rothman and Olney, 1986). Numerous studies have related ionotropic glutamate receptors to the regulation of cell survival, *in vivo* as well as *in vitro*. In most cases, exposure to agonists of glutamate receptors has been reported to lead to increased cell death, whereas antagonists were found to be protective. Whereas only N-methyl-D-aspartate (NMDA) receptors had been initially considered as possible actors in this domain (Regan and Choi, 1994; Lesort *et al.*, 1997, Drian *et al.*, 1999), it is presently clear that both kainic acid (KA) and amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors are also involved (Larm *et al.*, 1997; Solum *et al.*, 1997; Jensen *et al.*, 1998). Neurotoxicity initiated by overstimulation of glutamate receptors and subsequent influx of free Ca^{2+} leads to an intracellular cascade of cytotoxic events (Choi, 1985). Ca^{2+}-dependent depolarization of mitochondria has been suggested to contribute to oxidative stress in neuronal injury, through the production of reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion or nitric oxide (NO) (Choi, 1992; Dykens, 1994; Whit and Reynolds, 1996). KA-induced excitotoxicity, mediated via KA receptors, is also known to be associated with the excessive release of glutamate that may underlie the pathogenesis of neuronal injury (Arias *et al.*, 1990; Sperk, 1994). KA may induce neuronal damage through the excessive production of ROS and lipid peroxidation (Ben-Ari, 1985; Bondy and Lee, 1993). Thus, KA has been used as a model agent for the study of neurotoxicity.

Primary cultures of granule neurons derived from cerebella of postnatal rats have been frequently used to study mechanisms of neuronal death as an *in vitro* model. This is in part due to the fact that these are endowed with glutamate receptors, and glutamate receptors-mediated excitotoxicity is believed to play a role in the pathophysiology of neurodegenerative diseases (Manev *et al.*, 1990).

Zizyphi Jujube Semen (ZJS), the dried seed of *Zizyphus jujuba* Mill. (Rhamnaceae), has been known to contain more than 10 different alkaloids (Han and Park, 1986). ZJS has been used as an analgesic and an anti-convulsant

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*Author for correspondence
Fax: +82-43-267-3150, E-mail: vepharm@chungbuk.ac.kr

in oriental countries such as Korea and China for centuries. In addition, ZJS can calm the mind, increase flesh and strength and cause a sedative effect. ZJS is also recommended in the case of insomnia (Chopra *et al.*, 1956). In recent laboratory investigations, ZJS showed a tranquilizing and hypnotic effect. It can significantly increase sleep time induced by hexobarbital or pentobarbital (Han and Park, 1986; Adzu *et al.*, 2002). It has been reported that the alkaloid fractions from the seeds are the active principles producing central inhibitory activity (Han and Park, 1986). The glycosides contained in this herb have been reported to show potent immunological adjuvant activity (Matsuda *et al.*, 1999).

In a recent study, we demonstrated that ZJS prevented NMDA-induced neurotoxicity (in submission). The present study examined whether ZJS has the neuroprotective action against KA-induced cell death in primarily cultured rat cerebellar granule neurons. These works may contribute to extending pharmacological actions of ZJS in the CNS. The methanol extract of ZJS exhibited significant protection from the excitotoxicity induced by KA. It was also examined the effect of ZJS on the KA-induced $[Ca^{2+}]_c$ elevation, glutamate release and ROS generation.

Materials and Methods

Materials – ZJS was purchased from an oriental drug store in Taegu, Korea, and identified by Professor K.-S. Song, Kyungbuk National University. KA, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), o-phthaldialdehyde (OPA), 2-mercaptoethanol, trypsin (from bovine pancreas), Dulbecco's modified Eagle's medium (DMEM), poly-L-lysine, amino acids for HPLC standard, cytosine 1-β-D-arabinofuranoside hydrochloride (cytosine arabinoside), 0.4% trypan blue solution (pH 7.4), and Fura 2-AM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6,7-Dinitroquinoxaline-2,3-dione (DNQX) was purchased from Tocris (Bristol, UK). 2,7-Dichlorodihydrofluorescin diacetate (H_2DCF-DA) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum and Jocklik-modified Eagles medium were purchased from Gibco (Logan, Utah, USA). All other chemicals used were of the highest grade available.

Preparation of methanol extract of ZJS – ZJS (300 g) were extracted three times in a reflux condenser for 24 h each with 2 L of 70% methanol. The solution was combined, filtered through Whatman NO. 1 filter paper, and concentrated using a rotary vacuum evaporator followed by lyophilization. The yield was about 10% (w/w).

Primary culture of cerebellar granule neurons – Cerebellar granule cells were cultured as described previously (Kim *et al.*, 2001). Briefly, 7 to 8-day-old rat pups (Sprague-Dawley) were decapitated, and the heads were partially sterilized by dipping in 95% ethanol. The cerebellum was dissected from the tissue and placed in Joklik-modified Eagle's medium containing trypsin (0.25 mg/ml). After slight trituration through a 5 ml pipette 5-6 times the cells were incubated for 10 min at 37°C. Dissociated cells were collected by centrifugation (1,500 rpm, 5 min) and resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), L-glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM), penicillin (40 U/ml), gentamicin (50 μg/ml) and 10% fetal bovine serum. The cells were seeded at a density of about 2×10^6 cells/ml into poly-L-lysine coated 12 well-plates (Corning 3512, NY, USA) for the measurements of cell death and glutamate release, glass cover slides for the measurements of $[Ca^{2+}]_c$, and coverslips (Fisher Scientific 12CIR, Pittsburgh, PA, USA) for the measurements of ROS. After 2 days incubation, growth medium was aspirated from the cultures and new growth medium containing 25 mM KCl and 20 μM cytosine arabinoside, to prevent proliferation of nonneuronal cells, was added. Cultures were kept at 37°C in a 7% CO_2 atmosphere.

Neurotoxicity experiments – KA and DNQX were solubilized in the incubation buffer described below. ZJS was dissolved in absolute ethanol with the concentration of 5 mg/ml and further diluted with the buffer. The final concentration of ethanol was 0.1%, and did not affect cell viability (data not shown). Neurotoxicity experiments were performed on neurons grown for 8-10 days *in vitro* on either 12-well culture plates or glass coverslips placed in culture dishes. The culture medium was removed and neurons were washed with a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgSO_4$, 11 mM bicarbonate and 10 mM glucose at pH 7.4. They were then incubated for 30 min in the same medium, and incubated for a further 6 h (unless otherwise indicated) in the presence of KA at 37°C. For every experiment, ZJS or DNQX was added 15 min prior to the exposure of cells to KA and was present in the incubation buffer during the KA exposure.

Analysis of cell viability

Trypan blue exclusion assay – After completion of incubation with KA (500 μM), the cells were stained with 0.4% (w/v) trypan blue solution (400 μl/well, prepared in 0.81% NaCl and 0.06% K_2HPO_4) at room temperature for 10 min. Only dead cells with a damaged cell membrane are permeable to trypan blue. The numbers of trypan blue-permeable blue cells and viable white cells were counted in 6 randomly chosen fields per well under a phase contrast microscope (Olympus IX70, Tokyo, Japan). ZJS and DNQX (10 μM) were pretreated 15 min prior to the KA treatment.

MTT colorimetric assay – This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductases (Berridge and Tan, 1993). Therefore, the amount of formazan produced is proportional to the number of viable cells. After completion of incubation with KA (500 μM), the culture medium was replaced by a solution of MTT (0.5 mg/ml) in serum-free growth medium. After a 4 h incubation at 37°C, this solution was removed, and the resulting blue formazan was solubilized in 0.4 ml of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm using microplate reader (Bio-Tek EL_X808, Vermont, USA). Serum-free growth medium was used as blank solution. ZJS (0.5 mg/ml) and DNQX (10 μM) were pretreated 15 min prior to the KA treatment.

Measurement of $[Ca^{2+}]_C$ – Cells grown on glass cover slides were loaded with 5 μM Fura 2-AM [dissolved in dimethyl sulfoxide (DMSO)] for 40 min in serum-free DMEM at 37°C in the CO_2 incubator, and washed with the incubation buffer. Cell culture slides were mounted into spectrophotometer cuvette containing 3 ml incubation buffer. Fluorescence was measured with a ratio fluorescence system (Photon Technology International, RatioMaster^{TM}, NJ, USA) by exciting cells at 340 and 380 nm and measuring light emission at 510 nm. Baseline of $[Ca^{2+}]_c$ was measured for 120 sec prior to the addition of KA (50 μM). In order to test the effects of ZJS (0.5 μg/ml) and DNQX (10 μM) on KA-induced $[Ca^{2+}]_c$ change, the cells were exposed to the compounds in the incubation buffer for 15 min, after being loaded with Fura 2-AM and washed. The compounds were also present in the cuvette during the measurement of KA-induced $[Ca^{2+}]_c$ change. KA was applied into the cuvette through a hole using a micropipette and mixed by an attached magnetic stirring system. The increase of $[Ca^{2+}]_c$ was expressed as the fluorescence intensity ratio measured at 340 nm and 380 nm excitation wavelength (F340/F380). This experiment was carried out in the dark.

Measurement of glutamate concentration – After completion of incubation with KA (500 μM) for 6 h, glutamate secreted into the medium from the treated cells was quantified by high performance liquid chromatography with an electrochemical detector (ECD) (BAS MF series, Indiana, USA) (Ellison *et al.*, 1987). Briefly, after a small aliquot was collected from the culture wells, glutamate was separated on an analytical column (ODS2; particle size, 5 μm; 4.6×100 mm) after pre-derivatization with OPA/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ml/min. ZJS (0.5 μg/ml) and DNQX (10 μM) were pretreated 15 min prior to the KA treatment.

Measurement of ROS generation – The microfluorescence assay of 2',7'-dichlorofluorescin (DCF), the fluorescent product of H_2DCF-DA, was used to monitor the generation of ROS (Gunasekar *et al.*, 1996). Cells, grown on coverslips, were washed with phenol red-free DMEM 3 times and incubated with the buffer at 37°C for 30 min. Then, the buffer was changed into the incubation buffer containing 500 μM KA, and the cells were incubated for a further 1 h. In order to test the effects of ZJS (0.5 μg/ml) and DNQX (10 μM) on KA-induced generation of ROS, the compounds were added 15 min prior to the treatment with KA. The uptake of H_2DCF-DA (final concentration, 5 μM) dissolved in DMSO was carried out for the last 10 min of the incubation with KA. After washing, coverslips containing granule cells loaded with H_2DCF-DA were mounted on the confocal microscope stage, and the cells were observed by confocal scanning laser microscopy (Bio-rad, MRC1021ES, Maylands, England) using 488 nm excitation and 510 nm emission filters. The average pixel intensity of fluorescence was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer. The challenge of H_2DCF-DA and measurement of fluorescence intensity was performed in the dark.

Statistical analysis – Data were expressed as mean±SEM and statistical significance was assessed by the unpaired student *t-test.*

Results

ZJS protects neurons from toxicity induced by KA – Cell death after plasma membrane damage was assessed with the ability of cerebellar granule neurons to take up trypan blue. The trypan blue assay that detects multiple forms of cell death, including apoptosis or necrosis, has been used as an initial non-specific indicator of cell death. The number of cells stained by trypan blue with plasma membrane damage significantly increased with exposure of the cells to KA. In control cultures, the number of trypan blue-negative cells reached 93.0±1.9%, while the value decreased to 37.5±2.5% with the treatment with 500

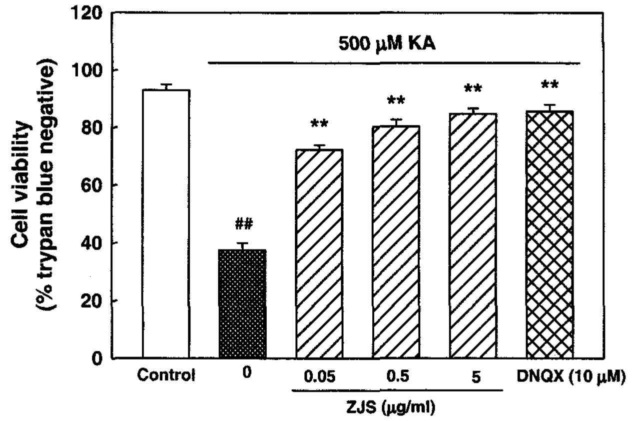

Fig. 1. Inhibitory effect of ZJS on KA-induced cell death in cultured cerebellar granule neurons. Neuronal death was measured by the trypan blue exclusion test. Results are expressed as mean±SEM values of the data obtained from four independent experiments performed in 2 or 3 wells. ##p<0.01 compared to control. **p<0.01 compared to 500 µM KA.

µM KA. ZJS showed concentration-dependent inhibition on the increase of neuronal cell death induced by KA (500 µM) over a concentration range of 0.05 to 5 µg/ml, showing 84.8±1.9 % with 5 µg/ml (Fig. 1). DNQX (10 µM), a KA receptor antagonist, also caused a marked inhibition of KA-induced neuronal cell death. For the following experiments, the concentration of 0.5 µg/ml for ZJS was used for the determination of the protective effects on the KA-induced neuronal cell damage.

As an additional experiment to assess KA-induced neuronal cell death, the MTT assay was performed. The MTT assay is extensively used as a sensitive, quantitative and reliable colorimetric assay for cell viability. When cerebellar granule neurons are exposed to 500 µM KA, the MTT reduction rate decreased to 51.4±2.5%. ZJS (0.5 µg/ml) significantly reduced the decrease of cell viability induced by KA, showing 81.2±4.0% (Fig. 2). Similarly, DNQX (10 µM) significantly inhibited the decrease of the MTT reduction rate caused by 500 µM KA.

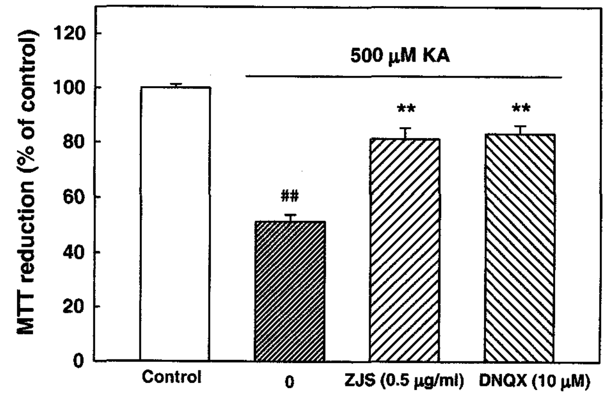

Fig. 2 Inhibitory effect of ZJS on KA-induced cell death in cultured cerebellar granule neurons. Neuronal death was measured by the MTT assy. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean±SEM values of the data obtained from four independent experiments performed in duplicate. ##p<0.01 compared to control. **p<0.01 compared to 500 µM KA.

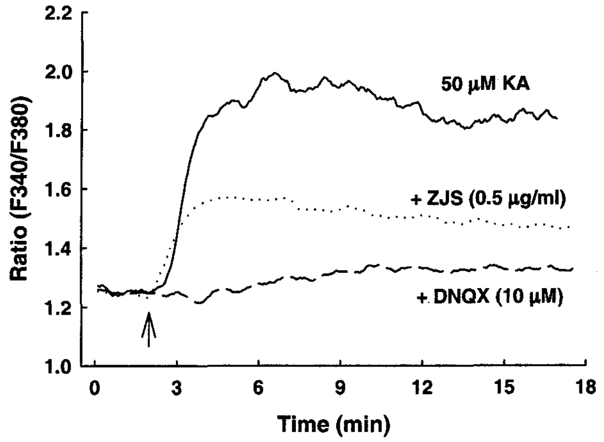

Fig. 3. Change of $[Ca^{2+}]_c$ in response to KA in the presence or absence of ZJS and DNQX in cultured cerebellar granule neurons. $[Ca^{2+}]_c$ was monitored using a ratio fluorescence system. In the plots shown, each line represents F340/F380 ratio from a representative cell population.

ZJS inhibits KA-induced elevation of $[Ca^{2+}]_c$ – The increase of $[Ca^{2+}]_c$ 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 cells is proportional to $[Ca^{2+}]_c$. As shown in Fig. 3, $[Ca^{2+}]_c$ started to elevate immediately after the treatment with 50 µM KA and reached maximal fluorescence intensity after 3-4 min. In contrast, KA application in the presence of DNQX (10 µM) failed to produce the increase of $[Ca^{2+}]_c$ throughout the measurement period. ZJS (0.5 µg/ml) significantly, but not completely, inhibited the KA-induced $[Ca^{2+}]_c$ elevation. ZJS or DNQX did not affect basal $[Ca^{2+}]_c$ (data not shown).

ZJS inhibits KA-induced elevation of glutamate release – Glutamate released into the extracellular medium was quantified after the incubation of cells with 500 µM KA for 6 h. As shown in Fig. 4, 500 µM KA markedly elevated the basal glutamate level from 0.33±0.07 to 1.89±0.08 µM and ZJS (0.5 µg/ml) strongly blocked the KA-induced elevation of glutamate release showing 0.79± 0.08 µM. In addition, DNQX (10 µM) markedly inhibited KA-induced elevation of glutamate.

ZJS inhibits KA-induced ROS generation – KA increased glutamate release and the cytosolic concentration of free Ca^{2+}. Furthermore, the pathological condition induced by glutamate is associated with accelerated formation of ROS. In H_2DCF-DA-loaded cerebellar granule cells, KA increased the fluorescence intensity, indicating

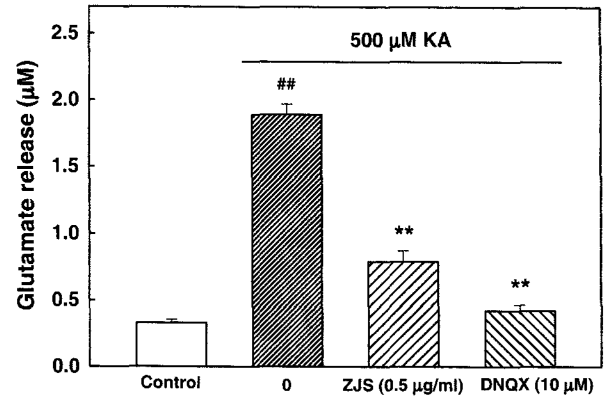

Fig. 4. Inhibitory effects of ZJS on KA-induced glutamate release in cultured cerebellar granule neurons. The amount of released glutamate was measured by HPLC with ECD. Results are expressed as meanSEM values of the data obtained in four independent experiments performed in 2 or 3 wells $^{\#\#}p<0.01$ compared to control. ** $p<0.01$ compared to 500 μM KA.

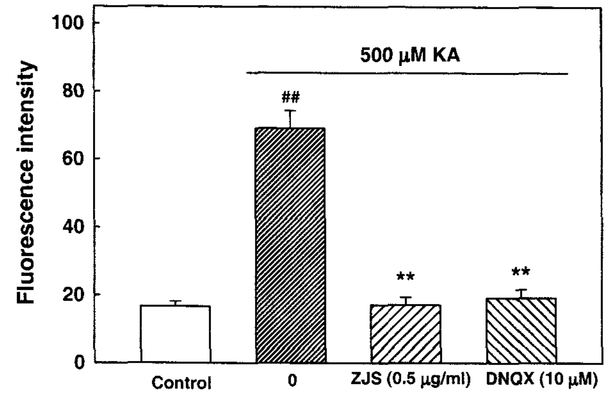

Fig. 5. Inhibitory effects of ZJS on KA-induced ROS generation in cultured cerebellar granule neurons. Values represent mean±SEM of relative fluorescence intensity obtained from four independent experiments performed in 2 or 3 wells. $^{\#\#}p<0.01$ compared to control. ** $p<0.01$ compared to 500 μM KA.

the generation of ROS. The fluorescence intensity in 500 μM KA-treated cells was increased more than four fold to 69.1±5.2 compared to control cells of 16.7±5.2. ZJS (0.5 μg/ml) and DNQX (10 μM) significantly blocked KA-induced increase in fluorescence intensity (Fig. 5). ZJS did not show direct reaction with H_2DCF-DA to generate fluorescence.

Discussion

Most of the previous hypotheses dealing with neurodegenerative diseases have invoked abnormal release and/or decreased uptake of the excitatory amino acid glutamate as playing a key role in the process of excitotoxicity. The neuronal death in such conditions as ischemia, hypoglycaemic coma, cerebral trauma or action of neurotoxins appears to be mediated at least in part by the extensive release of glutamate and its interaction with receptors (Coyle and Puttfarcken, 1993). The released glutamate, acting on glutamate receptors, secondly triggers Na^+ influx and neuronal depolarization. This leads to Cl^- influx down its electrochemical gradient, further cationic influx and osmotic lysis of the neuron, resulting in neuronal cell death (Van Vliet *et al.*, 1989). There is a great deal of data which shows that activation of the NMDA receptors elevates the influx of Ca^{2+} and non-NMDA (AMPA and KA) receptors promote the influx of Na^+, which can lead to membrane depolarization. In turn, depolarization can activate membrane voltage-sensitive Ca^{2+} channels, leading to additional Ca^{2+} influx. Many studies have shown that KA-induced elevation of $[Ca^{2+}]_i$ plays a fundamental role in the process of excitotoxicity (Choi, 1985; Weiss and Sensi, 2000). A sustained increase in $[Ca^{2+}]_i$ triggers a series of events including the elevation of cGMP, the glutamate release and the activation of NOS (Mei *et al.*, 1996; Baltrons *et al.*, 1997). Released glutamate secondly acts on glutamate receptors and therefore potentiates the neurotoxicity. KA-induced neurotoxicity is blocked by KA antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and DNQX, and Ca^{2+} channel antagonists (Carroll *et al.*, 1998; Simonian *et al.*, 1996; Weiss *et al.*, 1990). NOS inhibitors significantly reduce the KA-induced cell death in cell culture systems (Brorson *et al.*, 1994).

In the present study, long-term treatment with KA (500 μM) produced neuronal cell death in cultured rat cerebellar granule cells, in accordance with many previous reports. KA caused significant elevation of $[Ca^{2+}]_c$, glutamate release and ROS generation. This neurotoxicity induced by KA was completely reversed by DNQX, indicating that the neurotoxicity was mediated by the activation of the receptor. ZJS showed the concentration-dependent protection on KA-induced neuronal cell death, and blocked the KA-induced increase of $[Ca^{2+}]_c$, glutamate release and ROS generation.

The elucidation of the variety of events occurring downstream of neuronal Ca^{2+} overloading is still a matter for further research. ROS generation undoubtedly takes place in glutamate neurotoxicity (Pereira and Oliveira, 2000) and is likely due to Ca^{2+} influx in the cytosol. Ionotropic glutamate receptor agonists have been reported to increase the rate of ROS formation in an isolated synaptoneurosomal fraction derived from rat cerebral cortex (Bondy and Lee, 1993; Giusti *et al.*, 1996). Long glutamate treatment results in permanent damage of mitochondria and large uncoupling, which occurs simultaneously with high mitochondrial ROS production. In this case, cytosolic Ca^{2+} deregulation is followed by membrane permeability transition (Nicholls and Budd; 2000). In contrast with many reports that Ca^{2+} signals activate enzymes which are associated in ROS generation (e.g. xanthine oxidase, nitric oxide synthase, phospholipase A2) leading to lipid peroxidation and neuronal damage, it has been demonstrated that ROS generation can facilitate $[Ca^{2+}]_i$ increase by damaging the $[Ca^{2+}]_i$ regulatory mechanism and activating Ca^{2+} release from intracellular Ca^{2+} stores (Duffy and MacViar, 1996). It was not elucidated whether ZJS suppressed ROS generation through the inhibition of $[Ca^{2+}]_c$ increase, or vice versa, in the present study. However, a significant increase of ROS generation was not produced in 30 min after KA treatment (data not shown), indicating that ROS generation is a secondary event following $[Ca^{2+}]_c$ increase. Therefore, it was presumed that the neuroprotective effects of ZJS were mainly due to the inhibition on KA-induced elevation of $[Ca^{2+}]_c$, as shown in many compounds having the CNS inhibitory activities due to their inhibition on neuronal depolarization, and then this effect was followed by the inhibition on ROS generation and glutamate release. In addition, further study is necessary to clarify whether ZJS inhibits Ca^{2+} entries from extracellular medium or Ca^{2+} release from intracellular stores in cultured cerebellar neurons. The possibility of ZJS to bind to glutamate receptors could be excluded, since ZJS did not affect the specific $[^3H]$MK-801 binding to the cells (data not shown).

In conclusion, we demonstrated a novel pharmacological action of ZJS in the present study. The results strongly suggest that ZJS might be of value in preventing various neurodegenerative pathophysiological conditions.

Acknowledgements

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