Protective Effects of Kamidojuk-san on the Nervous Systems

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Kamidojuk-San (KDJS) is known to be effective for treating cardiovascular diseases such hypertension, and clinically applied for the treatment of cerebral palsy or stoke patients. Yet, the overall mechanisms underlying its activity at the cellular levels are not known. Using experimental animal system, we investigated whether KDJS has protective effects on cells in cardiovascular and nervous systems. KDJS was found to rescue death of cultured primary neurons induced by AMPA, NMDA and kainate as well as BSO and Fe^{2+} treatments. Moreover, KDJS treatment promoted animal's recovery from coma induced by a lethal dose of KCN treatment, and improved survival in animals exposed to lethal dose of KCN. Neurological examinations further showed that KDJS reduced the time which is required for animals to respond in terms of forelimb and hindlimb movements. To examine its physiological effects on cardiovascular and nervous systems, we induced ischemic injury in hippocampal neurons and cerebral neurons by middle cerebral artery (MCA) occlusion. Histological examination revealed that KDJS significantly protected neurons from ischemic damage. Thus, the present data suggest that KDJS may play an important role in protecting cells of cardiovascular and nervous systems from external noxious stimulations.

Key words : Kamidojuk-San, protective effects, MCA, ischemic damage

Introduction

Kamidojuk-San (KDJS) is known to be effective for treating cardiovascular diseases such hypertension, and clinically applied for cerebral palsy or stoke patients[1]. When the brain tissues are exposed to exogenous insults such as ischemic injury and hemorrhage, certain brain tissues or neuronal cell types are more susceptible to ischemic injury than others[3,6]. For example, hippocampal pyramidal cells, cerebellar Purkinje cells, or neurons in the globus pallidus of the basal ganglia structure are known to be more easily damaged. Although molecular mechanism is not completely understood, changes in the levels and metabolism of certain neurotransmitters (e.g., glutamate) have been suggested.

Hypoxic ischemia is the most common cause of ischemic damage although brain damage by imperfect perfusion is often more serious than hypoxia alone. Oxygen depletion can cause loss of active pumps in neurons, resulting in depolarization of neurons followed by excitotoxic damage of postsynaptic neurons by neurotransmitters[15,34]. Deleterious effects by excitatory amino acids (e.g., glutamate in the central nervous system) include cytotoxicity by prolonged, elevated levels of calcium, free radicals, and by activation of molecular machineries of apoptotic pathway[21]. Indeed, treatment of glutamate or its analogues kainate or NMDA can induce death of cultured primary neurons. Similarly, these agents, when introduced into the brain, can cause death of neuronal cell, and also result in pathophysiological abnormalities such as stroke, or epilepsy[7,28,32]. Since hypoxic-ischemic excephalopathies, along with intracranial hemorrhage, are the most fatal vascular diseases in the nervous system it would be critical to understand molecular responsiveness in brain tissues after ischemic insults. In this aspects, several caspases which are activated during apoptotic cell death are one of the promising candidates to regulate cell death[19].

Although the central nervous system is generally known to be immune-privileged, several studies have indicated that the permeability of blood brain barrier increases during ischemic damage[1-2], and autoimmune damage can occur in the brain[31,33]. This further implies that inflammation in the brain increases infiltration of immune cells (macrophage and cytotoxic T cells). It is therefore important to maintain normal composition of blood; In other words, inclusion of inflammatory cytokines, steroid hormones, or catecholamines

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hormones in blood can be transported into the parenchymal cells in the brain in certain condition, and cause hypersensitivity reactions in neurons. Also, abnormal regulation of membrane potential by prolonged stimulation of neuronal cells by high K^+ or abnormal increases in intracellular free radicals can lead to deteriorating hyperexcitability of postsynaptic neurons. Since these and other factors are all fatal to cells exposed to noxious stimulation, one of the promising approaches for the treatment of brain ischemia would be to examine drugs which might affect on multiple signaling pathways in nervous system as well as other organ systems (cardiovascular and immune system).

In the present study, we examined whether KDJS has any protective effects from exogenous insults on the brain tissues. By using cultured primary cortical neurons, possible neuroprotective effects of KDJS on neuronal cells given to cytotoxic insults were examined. Our data showed that KDJS reduced neuronal cell death induced by AMPA, NMDA or kainate. It was also found that KDJS has a protective role in brain tissues from ischemic insults by middle cerebral (MCA) occlusion. Our results therefore suggest that KDJS may play an important role in protecting neuronal cells from a diverse rage of external insults in brain.

Materials and Methods

1. Materials

1) Experimental animals

Sprague-Dawley rats (180-200 g female) and mice (albino ICR, 18-20 g) were purchased from the Korea Experimental Animal Center. Animals were fed with food pellets (; composed of crude proteins 21 %, crude fats 8 %, crude carbohydrates 5 %, minerals 8 %, Calcium 0.6 %, Phosphorous 0.4 %; Samyang Animal Food Co.) and water, and adapted for at least 2 weeks before the experiment in the animal room with 22 ± 2 ℃, relative humidity of 50 ± 10 %, with 12 hours of day and night cycle and an luminescence of 150 - 300 Lux. Only healthy animals showing normal body weight increase were used for the experiment.

2) Drugs

Kamidojuk-San used in the present study was obtained from Daejeon University Oriental Medicine Hospital. One seal of KDJS has the composition as shown in the Table 1.

3) Chemicals and Instruments

Chemicals were obtained from following sources; KCN (Potassium cyanide; Sigma Co., U.S.A.), Dulbecco's phosphate buffered saline (DPBS-A; Sigma Co., U.S.A.), normal saline (Joong-Wei Pharmaceutical Co. Inc., Korea), sodium citrate (Sigma Co., U.S.A), 2,3,5-triphenyl-2H-tetrazoliumchloride (Sigma Co., U.S.A), gerorane (Enflurane reagent, Joong-Wei Pharmaceutical Co. Inc., Korea), xantopren VL (Bayer Dental, Japan), optosil-Xantopren activator (Bayer Dental, Japan), cresyl fast violet (Sigma Co., U.S.A.), paraformaldehyde (Sigma Co., U.S.A.), aldosterone RIA Diagnostic kit (Abbott Co., U.S.A.), superoxide dismutase (Stressgen Co., USA), histostain plus kit (Zymed Co., U.S.A), H_2O_2 (Sigma Co., U.S.A), formalin (Sigma Co., U.S.A), glutaraldehyde (Sigma Co., U.S.A), OsO_4 (Sigma Co., U.S.A), toluidine blue (Sigma Co., U.S.A), hematoxylin (Sigma Co., U.S.A), eosin (Sigma Co., U.S.A), NMDA (N-methyl-D-asparate, Sigma), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, Sigma), kainate (Sigma), Fe^{2+} (ferrous chloride, Sigma), BSO (buthionine sulfoximine, Sigma).

Experimental instruments and apparatus used in this study were as following; serum separator (Green Cross Co., Korea), minus-ST (Cobras Co., France), centrifuge (Beckman Co., U.S.A.), rotary vacuum evaporator (Buhl 461, Switzerland), deep freezer (Sanyo Co., Japan), freeze dryer (Eyelid Co., Japan), autoclave (Hirayama, Japan), ultrasonic cleaner (Branson ultrasonics Corp., U.S.A.), roller mixer (Gowon scientific technology Co., Korea), vortex (Vision Co., Korea), brain matrix (ASI Instrument, Warren, MI., U.S.A.), royal Multi-Plus (Royal Medical Co., Korea), camera (Nikon, Japan), ACL-100 (Instrumentation Laboratory, U.S.A.), physiograph Model 7 (GRASS Instrument Co., Quincy, Mass., U.S.A.), optical microscope (Olymphus BH-2., Japan).

Table 1. The Compositions of KDJS Extracts

生 藥 名	韓 藥 名	用 量(g)
Liriopis Tuber	麥門冬	8
Coptidis Rhizoma	黃蓮	8
Scutellariae Radix	黃芩	4
Pinelliae Rhizoma	半夏	4
Lycii Radicis Cortex	地骨皮	4
Paeonia Radix Rubra	赤芍藥	4
Akebiae Caulis	木通	4
Rehmanniae Radix	生地黃	4
Poria	白茯神	4
Glycyrrhizae Radix	甘草	4
Junci Medulla	燈心	1
Total amount		49

2. Methods

1) Preparation of KDJS reagent

Four seals of KDJS were suspended in 2 liter of water, heat-extracted for 3 hr, and filtered three times. The filtered fluid was distilled using the rotary vacuum evaporator. Concentrated solutions were frozen at 70 ℃ for 4 hr, and freeze-dried for 24 hr. The yield of the powder after freeze-drying was an average of 10.75 g per each seal, and

used for experiment by diluting with physiological saline solution.

2) Primary cortical neuron culture

Fetal cortices were dissected from mouse embryo (embryonic day 15). Tissues were dissociated, and 1 x 10^5/well were plated on a 24 well plate. The medium used were Eagle's minimal essential media (Eagle's salts, supplied glutamine-free) including 5 % horse serum, 5 % fetal bovine serum, 2 mM glutamine, and 21 mM glucose medium. The plate used in the study was precoated with 100 ㎍/㎖ poly-D-lysine and 4 ㎍/㎖ laminine. Cells were maintained in a CO_2 incubator at 37 °C, and ara C was added 3 - 5 days after plating and used for experiment on days in vitro (DIV) 7-10.

3) Analysis of excitotoxicity and oxidative stress

Excitotoxicity was studied in cultured cortical neurons by treating NMDA (20 μM), AMPA (20 μM), and kainate (50 μM). Oxidative stress was investigated by inducing cells in solutions containing Fe^{2+} (100 μM) and buthionine sulfoximine (10 mM). Cells were then further incubated for 24 hr in Eagle's minimal essential media containing glucose (21 mM) and bicarbonate (26.5 mM). Culture media were removed and used for cell death measurement by LDH

4) Induction of brain ischemia

(1) Induction of Global ischemia

Global ischemic model was essentially followed by Schubert method. KDJS (7.16 mg/20 g body weight) was given to ICR mice by oral injection once each day. Control group animals were administered with the same volume of saline vehicle. Thirty min later, sublethal dose of KCN (i.e., 1.87 mg/kg) was injected into the arteriole, and the time interval until the animals regained the orientation reflex was measured. Also, the time period of survival was determined after the injection of lethal dose of KCN (3.0 mg/kg).

(2) Middle cerebral artery (MCA) occlusion

Two hours before the experiment, SHRs were weighed, injected with KDJS (2 ml, 71.6 mg/200 g body weight) or the same volume of saline vehicle. Utilizing the Oral zonda (Daejong Instruments, Korea), animals were subject to MCA occlusion experiment followed by neurological examination. Silicon rubber was prepared by heating and making the knob at the one end of the nylon stitching fiber (4-0 size, Deknatel Inc., Japan). The fiber was cut into 18 mm length, and was inserted into the silicon rubber tubing which was pretreated with hardening agent. To induce focal ischemia, SHRs were anesthetized by inhaling enflurane in a mixture of N_2O and O_2 (7:3 ratio) in the Royal Multi-Plus apparatus (Royal Medical Co., Korea). Ischemia surgery was done as developed previously (Nagasawa). Briefly, the central area of the neck was incised, and the right total veins, internal veins, and external veins were exposed. Total veins and external veins were ligated. Then, the probe was inserted into the external and internal veins except the knob area, and the distal potions of vessels were ligated to occlude the ipsilateral MCA. Ligation surgery was done within 30 min, and the animals body temperature was maintained by illuminating infrared rays and monitored by thermometer. During the middle cerebral artery (MCA) occlusion, animal was exposed to hypoxic atmosphere (90 % N_2O plus 10 % O_2) for 60 min, and blood flow was regained by pulling out the probe by 1 cm length. Animal was sacrificed 24 hr later to prepare the brains. Coronal brain slices (2 mm thickness) were prepared using brain matrix (ASI Instrument, Warren, MI., USA). Eight slices were collected, treated with 2 % triphenyltetrazolium chloride (TTC) solution for 20 min at 37 °C. Normal tissues were stained with dark red color whereas ischemic tissues were not stained, which enabled to distinguish between them. After photography, the tissues were fixed with 10 % formalin neutral buffer. Ischemic area and edema ratio were calculated as follows.

$$\text{Ischemic area (\%)} = C/(A + B) \times 100$$

$$\text{Edema ratio (\%)} = (A + B)/(2 \times B) \times 100$$

A: Cerebral hemisphere area which induced ischemia in the slice
B: Total cerebral hemisphere area in the slice
C: Ischemic area in the slice

(3) Neurological examination

Neurological abnormalities in response to MCA occlusion were performed in animals after induction of hypoxia, or after regaining blood flow. Levels of neurological abnormalities were graded according to Bedersons method (Table 2). The forelimb movement was graded 1 - 4, and hindlimb was graded 1 and 2.

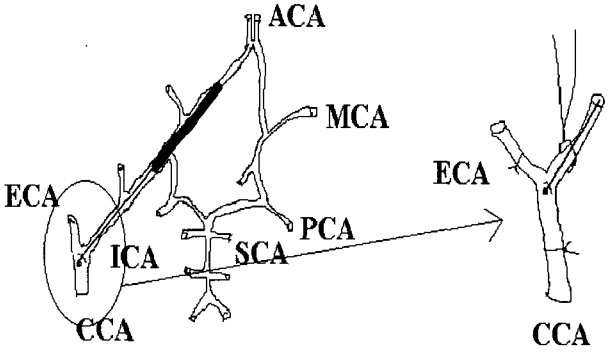


Fig. 1. Schematic representation of position of silicone rubber cylinder during occlusion of right middle cerebral artery(MCA). ACA, anterior cerebral artery; PCA, posterior cerebral artery; ICA, internal carotid artery; ECA; external carotid artery; CCA; common carotid artery, SCA, superior cerebral artery.

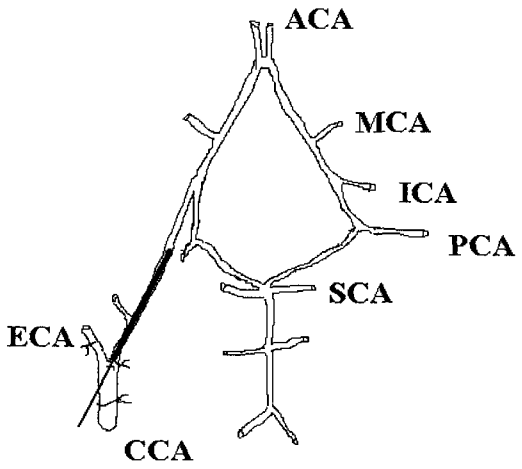


Fig. 2. Schematic representation of position of silicone rubber cylinder during occlusion of right middle cerebral artery(MCA). ACA, anterior cerebral artery; PCA, posterior cerebral artery; ICA, internal carotid artery; ECA; external carotid artery; CCA; common carotid artery, SCA, superior cerebral artery.

(iv) Histological observation

Brain tissues obtained from individual experimental groups were sliced into 2 mm thickness, stained with TTC, and fixed with 4 % paraformadehyde for 24 hr. Fixed tissues were washed with running tap water for 12 hr, and dehydrated with increasing concentrations of ethanol, histocleared, and then used for preparing paraffin blocks. The extent of neuronal damage was determined as follows. The paraffin blocks were cut into 4 um sections, followed by hydration and cresyl violet staining. After coverslipping, sections were observed under microscope (Olympus-BH2, Japan).

Table 2. The Neurologic Examination Grading System

	Grade	Neurologic examination
Forelimb	Grade 0	No deficit
	Grade 1	Forelimb flexation when suspended by the tail
	Grade 2	Reduced forepaw resistance to lateral push
	Grade 3	Circulating behaviour during suspension (body twisting)
Hindlimb	Grade 0	Immediate placement of the behind back on to the table (normal)
	Grade 1	No limb placement / movement

5) Statistical analysis

Number data among groups were compared by unpaired students t-test or analysis of variance (ANOVA) using SPSS/PC statistical program. A criterion for statistical significance was assessed at $p < 0.05$, $p< 0.01$, and $p < 0.001$.

Results

1. Neuroprotective effects of KDJS from cytotoxic stimulation; In vitro study

1) Neuroprotection from AMPA

In order to examine the neuroprotective effects of KDJS from excitotoxic insults, primary neuronal cells were prepared from brains tissues of newborn rats. Cells were cultured for two weeks and cell death was determined by LDH assay after various treatments. Optimal doses for inducing cell death were found to be 20 μg/ml, 20 μg/ml or 50 μg/ml for NMDA, AMPA, or kainate respectively.

Primary cortical neurons on DIV 13-15 were treated with NMDA to determine cytotoxicity. In saline treated animals, NMDA treatment (20 μg/ml) resulted 97.02±4.24 % of LDH release. In contrast, levels of LDH release were decreased in KDJS-treated neurons in a dose-dependent manner. The data were summarized in Table 3.

2) Neuroprotection from AMPA

Levels of cytotoxicity were investigated in AMPA-treated primary cortical neurons on DIV 13-15. In saline treated animals, LDH release was 54.01±2.11 % by 20 μM AMPA treatment. In contrast, KDJS treatment (10 - 300 μg/ml) decreased LDH release lower than 35 % (Table 4).

Table 3. Inhibitory Effect of KDJS on LDH Release Induced by NMDA

	NMPA(20 ㎍/ml)
alone	97.02±4.24 (%)
10	108.75±7.66 (%)
30	82.74±4.02 (%)
100	71.71±12.02 (%)
200	65.54±12.02 (%)

Table 4. Inhibitory Effect of KDJS on LDH Release Induced by AMPA

	AMPA (20 ㎍/ml)
alone	58.68±2.11 (%)
10	35.76±0.32 (%)
30	23.22±1.26 (%)
100	23.40±1.93 (%)
200	22.70±3.38 (%)

3) Neuroprotection from kainate

In the saline control animal group, kainate (50 μM) induced LDH release (65.10±5.94 %), whereas LDH release was reduced lower than 30 % in KDJS-treated group. Number data on the neuroprotective effects of KDJS were summarized in Table 5.

Table 5. Inhibitory Effect of KDJS on LDH Release Induced by Kainate

	Kainate (50 ㎍/ml)
alone	65.10±5.94 (%)
10	28.15±4.15 (%)
30	27.71±4.94 (%)
100	19.36±0.96 (%)
200	11.91±3.67 (%)

4) Cell protection from free radicals

(1) Primary cortical neurons on DIV 13-15 were treated with 10 mM BSO or equivalent volume of vehicle. In saline control animals, levels of LDH release were 46.80±5.81 (%) of vehicle control (0 ㎍/㎖ KDJS). In KDJS treated animal group, levels of LDH were decreased to 12.00±2.52 (%), 12.55±3.52 (%), and 6.53±0.53 (%), and 15.51±6.29 (%) for 10, 30, 100, 300 ㎍/㎖ of KDJS concentrations respectively. (Table 6).

Table 6. Inhibitory Effect of KDJS on LDH Release Induced by BSO

KDJS (㎍/ml)	BSO (10 mM)
0	46.80±5.81 (%)
10	12.00±2.52 (%)
30	12.55±3.52 (%)
100	6.53±0.53 (%)
300	15.51±6.29 (%)

(2) Cell protection from Fe^{2+} treatment

In cells treated with 100 μM Fe^{2+}, LDH release levels were 51.17±10.18 (%) whereas those in KDJS-treated animal group were decreased in a dose-dependent manner. Changes in LDH release by Fe^{2+} are summarized in Table 7.

Table 7. Inhibitory Effect of KDJS on LDH Release Induced by Fe^{++}

	Fe++(100 μM)
0	51.17±10.18 (%)
10	35.08±5.71 (%)
30	22.16±4.31 (%)
100	7.15±1.75 (%)
300	6.61±1.52 (%)

2. Cell protection of KDJS against ischemic injuries; In vivo study

1) Neuroprotective effects of KDJS on KCN-induced global ischemia

In KCN-treated animals, levels of animal's coma were first determined. Duration of coma in albino ICR mice after treatment with sublethal dose of KCN (1.87 ㎎/㎏/20g i.v) was 20.57±4.45 sec. In mice treated with KDJS-treated animals, the duration (18.21±3.11sec) was shortened, but not statistically significant (Table 8, Fig 3).

Table 8. The Duration of KCN-induced (1.87㎎/㎏ i.v.) Coma 15 min. after Oral Administration of KDJS Extracts in ICR Mice

Group	No. of Animals	Duration of coma(sec; Mean ± Standard Error)
Control	10	20.57±4.45
KDJS	10	18.21±3.11

Control : 1.87㎎/㎏ KCN i.v. injected group after oral administration of normal saline
KDJS : 1.87 ㎎/㎏ KCN i.v. injected group after oral administration of 9.2㎎/20g of KDJS extracts

The survival rate of KDJS group was 20 % with a lethal dose of KCN (3.0 ㎎/㎏/20g i.v.) at which dose saline injected control animals were all dead (Table 9).

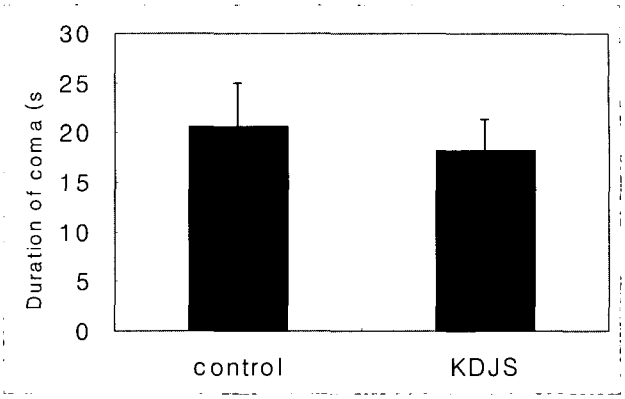


Fig. 3. The Duration of KCN-induced (1.87㎎/㎏ i.v.) Coma 15 min. after Oral Administration of KDJS Extracts in ICR Mice

Table 9. Number of animals survived after KCN-induced (3.0㎎/㎏ i.v.) coma in ICR Mice

Group	No. of Animals	No. of animals survived	% of Survival duration
Control	10	0	0
KDJS	10	2	20

Control : 1.87㎎/㎏ KCN i.v. injected group after oral administration of normal saline
KDJS : 1.87㎎/㎏ KCN i.v. injected group after oral administration of 9.2㎎/20g of KDJS extracts

2) MCA occlusion tests

Ischemic area and edema rate by MCA occlusion were determined in KDJS-treated and control Sprague-Dawley rats. Ischemic areas by MCA occlusion in KDJS-treated animals were significantly reduced compared to saline control group (Table 10, Fig 4, 6). Edema rate was significantly reduced in KDJS-treated groups as well (Table 11, Fig 5, 6).

Table 10. Ischemic Ratio in MCA Occluded SD Rats

No. of slices (n=4)	The mean area of infarction(%) control (Mean ± Standard Error)	The mean area of infarction (%) sample KDJS
1	20.78 ± 4.20	0*
2	36.30 ± 2.35	0
3	38.71 ± 4.68	0
4	32.25 ± 2.62	0
5	25.24 ± 5.36	0
6	8.70 ± 3.24	0
7	4.01 ± 2.08	0
8	0	0

Control : Oral administration of normal saline extracts, 0* : Not detectable
KDJS : Oral administration of 92.5㎎/200g of KDJS extracts

3) Neurological examination

In the forelimb test, the movement scores in the control group were 3.00±0.00, 2.25±0.25, 2.5±0.28 (Table 12). In KDJS treated group, the scores were improved showing 1.00±0.00, 1.5±0.25, 1.75±0.25. In the hindlimb test, the scores in the control group were 1.00±0.00, 1.00±0.00, and 1.00±0.00 (Table 13). In KDJS treated group, the scores were improved revealing 0.75±0.5, 0.00±0.00, and 0.5±0.57 (Table 13).

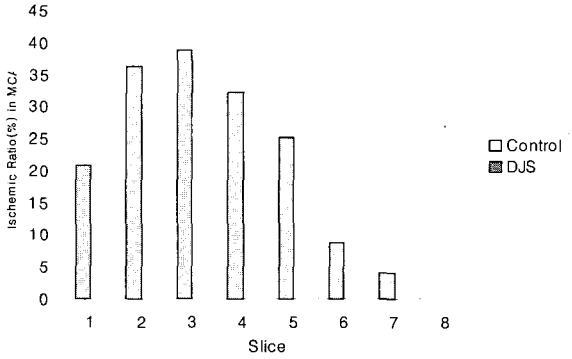


Fig. 4. Ischemic Ratio in MCA Occluded SD Rats. Control : Oral administration of normal saline extracts. KDJS : Oral administration of 92.5㎎/200g of KDJS extracts. Note that Values of the ischemic ratio in KDJS-treated animals were all '0', therefore not displayed in the Figure.

Table 11. Edema Ratio in MCA Occluded SD Rats

No. of slices (n=4)	The mean extent of edema(%) control	The mean extent of edema(%) sample KDJS
1	11.71 ± 3.56	0
2	20.87 ± 5.28	5.70 ± 2.24
3	17.03 ± 3.26	7.20 ± 4.67
4	19.15 ± 4.79	8.44 ± 2.0
5	7.35 ± 1.89	3.84 ± 3.26
6	1.17 ± 3.86	0
7	0	0
8	0	0

a) : Mean ± Standard Error, Control : Oral administration of normal saline extracts, KDJS : Oral administration of 92.5㎎/200g of KDJS extracts, Note that Values of the ischemic ratio in KDJS-treated animals were all '0', therefore not displayed in the Figure.

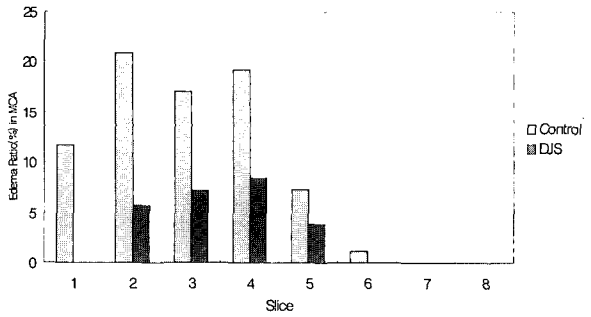
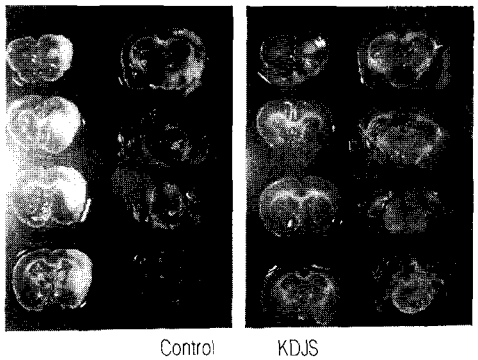


Fig. 5. Edema ratio in MCA occluded SD rats. Control : Oral administration of normal saline extracts. KDJS : Oral administration of 92.5㎎/200g of KDJS extracts



Control KDJS

Fig. 6. Ischemic and edema in MCA occluded SD rats. Control : Oral administration of normal saline extracts. KDJS : Oral administration of 92.5/200g of KDJS extracts

Table 12. Forelimb Test of Rat Treated in KDJS

Sample	Operation	Hypoxia	Recirculation
Control	3.00±0.00	2.25±0.25	2.5±0.28
KDJS	1.00±0.00	1.00±0.00	1.00±0.00

a) : Mean ± Standard Error. Control : Oral administration of normal saline extracts. KDJS : Oral administration of 92.5㎎/200g of KDJS extracts

Table 13. Hindlimb Test of Rat Treated in KDJS

Sample	Operation	Hypoxia	Recirculation
Control	1.00±0.00	1.00±0.00	1.00±0.00
KDJS	0.75±0.25	0.75±0.25	0.5±0.28

a) : Mean ± Standard Error. Control : Oral administration of normal saline extracts. KDJS : Oral administration of 92.5㎎/200g of KDJS extracts

4) Histological examination of hippocampal tissues after MCA occlusion

In normal animals, the six-layered organizations of cortical structures were clearly observed. Particularly strong staining was observed in the large pyramidal cells in layer 3 and 5. In the hippocampus, stronger staining was observed in the pyramidal cells and weaker staining in round-shape cells in the CA1-3 pyramidal cells (Fig 7).

In animals which were subject to MCA occlusion, six-layered cortical structures and stronger staining of large pyramidal cells in layer 3 and 5 were clearly observed in the normal side of the hemisphere. In contrast, in the side of ischemic insults, layered structures were not clear, nuclei were shrunken, the border between cells was unclear, and various sizes of vacuoles were observed (Fig 8).

In the hippocampus of animals treated with KDJS along with MCA, all cell layers including dentate granule cells and pyramidal cells were clearly shown. In the side of ischemic insults, pyramidal cells in the hippocampal formation were not clearly shown, but the granular shape of cells and vacuoles was observed. (Fig 9).

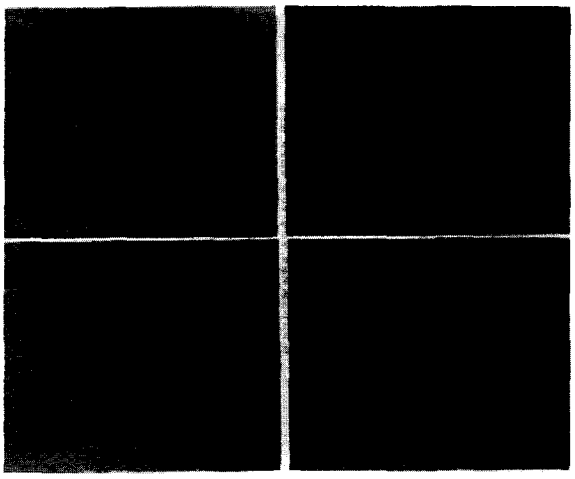


Fig. 7. Light micrographic appearance of the cerebral cortex and hippocampus, the normal group and cresyl fast violet stain, X100. A. Normal cerebral cortex portion B. Ischemic cerebral cortex portion C. Normal hippocampal formation portion D. Ischemic hippocampal formation portion

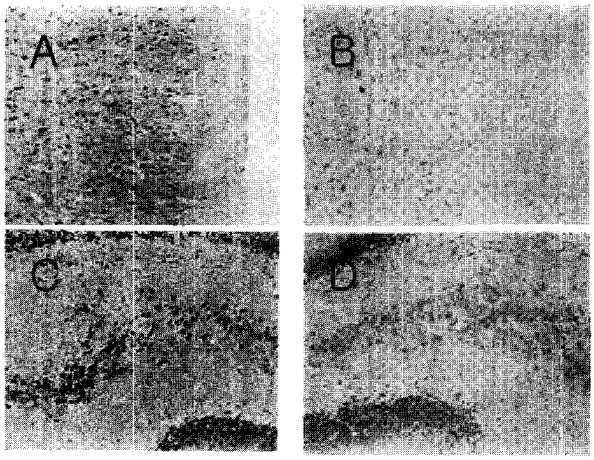


Fig. 8. Light micrographic appearance of the cerebral cortex and hippocampus, the control group and cresyl fast violet stain, X100. A. Normal cerebral cortex portion B. Ischemic cerebral cortex portion C. Normal hippocampal formation portion D. Ischemic hippocampal formation portion

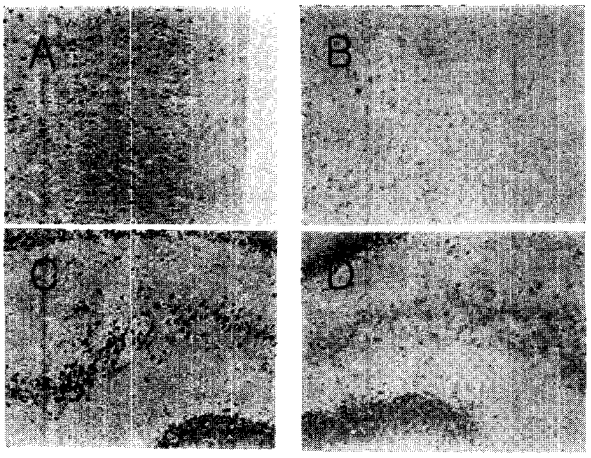


Fig. 9. Light micrographic appearance of the cerebral cortex and hippocampus, the KDJS group and cresyl fast violet stain, X100. A. normal cerebral cortex portion B. ischemic cerebral cortex portion C. normal hippocampal formation portion D. ischemic hippocampal formation portion

Discussion

Kamidojuk-San(KDJS) is known to be effective for treating cardiovascular diseases such hypertension, and clinically applied for cerebral palsy or stoke patients. In the present study, we found that KDJS protects neuronal cells from cytotoxic insults, which might be mediated by lowering the cell's responsiveness to noxious stimulations such as excitotoxic amino acids or free radicals in neuronal cells.

The KDJS is a mixture containing more than 10 kinds of herb, and it was implicated that its effects on regulating cell fate would be complicated, as would be case for the most of herb prescriptions. Potential protective effects of KDJS were investigated using both in vitro culture system and in vivo experimental animal models. Excitatory neurotransmitters glutamate analogues resulted high levels of neuronal death, but cells pretreated with KDJS were significantly protected from excitotoxic death. KDJS, when treated with increasing does of NMDA, AMPA, or kainate up to 200 μg/ml rescued cells from death measured by LDH release. High doses of NMDA, AMPA, and kainate are known to cause cell damage by prolonged excitability in postsynaptic neurons[16-17,24]. Prolonged increases in cytoplasmic calcium[18] and reactive oxygen species and others[25] are believed to cause cell damage.

Yet, because mechanisms of neurotoxicity mediated by NMDA, AMPA, or kainate are not the same, possible protective effect of KDJS on neuroprotection remains to be further investigated.

NMDA, AMPA, and kainate activate their specific subtypes of glutamate receptors in neuronal cells. Extensive studies on characterizing glutamate receptors have revealed clear differences in channel opening properties between NMDA receptor and AMPA/kainate receptors (non-NMDA receptor). Non-NMDA receptor accounts entirely for fast excitatory postsynaptic potential (EPSP) by allowing sodium inflow into the postsynaptic spine. In contrast, the NMDA receptors have unusual permeability properties. NMDA receptors are not activated at the resting membrane potential even in the presence of agonist because Mg ions block the pore, then when the potential increases to -50 mV, receptors are activated to increase calcium inflow. Numerous studies further indicated that the activation of NMDA receptors are related to synaptic plasticity which are believed to occur during learning and memory. In the hippocampus, activation of NMDA receptors is required for the induction of long-term potentiation (LTP), an experimental model of learning and memory[4,20]. NMDA specifically binds to NMDA receptors, and causes depolarization of postsynaptic membrane and calcium inflow[4]. Increased calcium inflow is important as a second messenger for the maintenance of LTP by modifying proteins such as calcium/calmodulin kinase II, AMPA receptors, and others[20]. However, excess NMDA stimulation was shown to cause neuronal cell damage, which is thought be mainly due to prolonged increases in cytosolic calcium in the posysynaptic neurons[7,15,25]. Neurotoxic effects of kainate have been well demonstrated by histological observation of hippocampal pyramidal cells, and by the occurrence of seizure onset, indicating that kainate is very potent[3].

Despite the differences in cytotoxicity by NMDA, AMPA, and kainate as described above, several studies have demonstrated increased production of free radicals in the cytoplasm as a common denominator causing cell damage. Consistent with this possibility is our data which showed KDJS-mediated neuroprotection from BSO- or Fe^{2+}-treated

cortical neurons. BSO- and Fe^{2+} are known to increase the production of free radicals[8,10,13,27,35]. Further studies to identify chemical compositions of KDJS are essentially the first critical step to understand molecular mechanisms underlying KDJS-mediated neuronal protection.

The protective effect of KDJS was also demonstrated using in vivo ischemic injury model. As an experimental model of global ischemia, neurotoxic agent KCN[11] was injected into rats which were pretreated with KDJS or saline vesicle. In cells, reducing power generated from citric acid cycle is transported into the mitochondrion and used for generating APT via the oxidative phosphorylation, and cyanide blocks the electron transfer, causing cytotoxicity. It was noted that KDJS treatment with lethal dose improved animal's survival. Moreover, when sublethal dose of KCN was given, the recovery time from coma was faster in KDJS-treated animals than control animals. These data suggest that KDJS may play a role in protecting neuronal cells from ischemic damage.

Although any specific neuronal tissue/cell type indicating the better capacity of recovery cannot be defined at this moment, cells having high metabolic activity could be primarily the candidate because neurotoxic insults by KCN block respiratory function.

We also found that KDJS treatment protected hippocampal pyramidal neuron and cerebral pyramidal neurons from ischemic damage caused by the MCA occlusion. The MCA occlusion is the widely accepted, experimental model of ischemic injury[9,14,22], and neurons are one of the most susceptible targets of ischemic injury[5,23,26]. Oxygen deficiency caused by ischemia results in reduced production of ATP in the mitochondria and increased cytoplsmic calcium concentration which can activate protease and phospholipase activity. Inactivation of Na/K pump due to limited supply of ATP, breakdown of cell components by proteases and lipases are all combined to cause neuronal depolarization, rendering neuron vulnerable to excitatory stimulation. It should also be noted that if the presynaptic neurons are depolarized in a similar manner, there would be an increased release of neurotransmitters from the presynaptic terminal. All these can further increase cytotoxic damage[15]. While neuronal damage causes cell death primarily by necrosis, several lines of recent studies indicate that ischemic insults can activate caspase-activated apoptotic pathway[19,29]. We found that in KDJS-treated animals, mean ischemic ratio and the extent of edema were significantly reduced after ischemic injury. Also, KDJS was found to protect hippocampal pyramidal neurons and cerebral pyramidal neurons. Since these tissues are important for several brain functions such as learning and memory, programmed motor balances, and cognitive behavior, further KDJS studies in relation to behavioral activity in animals will be interesting. In relation to this issue, our measurements of consciousness test by forelimb and hindlimb reflex scores indicate positive effects of KDJS. Since KDJS was shown to reduce excitotoxic amino acid (EAA)-mediated toxicity (see above), we speculate that neuronal cells in vivo might be able to protect cells from damage related to prolonged neuronal cell depolarization.

Conclusion

By using several experimental approaches, we have investigated the potential protective role of KDJS in cells of cardiovascular and nervous systems. KDJS appears to protects cells in both neuronal and non-neuronal system from various kinds of external noxious stimulations. Further studies are required to examine molecular mechanism on which molecular components act on reducing cytotoxicity. To do this, it would be, first of all, critical to analyze the chemical compositions of KDJS. It should be also kept in mind that some components of KDJS might have adverse effects which need to be carefully sorted out and eliminated from the prescription for the purpose of basic research as well as clinical applications.

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