Kami-bang-pung-tong-sung-san is Involved in Protecting Neuronal Cells from Cytotoxic Insults

Young Cheul Na, Gung Uk Nam[1], Yong Koo Lee[2], Dong Hee Kim*

Department of Pathology, College of Oriental Medicine, 1:Department of Neurophysiology, College of Oriental Medicine, 2:Department of Western Internal Medicine, College of Oriental Medicine, Daejeon University

KBPTS is the fortified prescription of Bang-pung-tong-sung-san (BPTS) by adding *Spatholobi Clulis* and *Salviae Miltiorrzae Radix*. BPTS prescription has been used in oriental medicine for the treatments of vascular diseases including hypertension, stroke, and arteriosclerosis, and nervous system diseases. Yet, the overall mechanism underlying its activity at the cellular levels remains unknown. To investigate the protective role of KBPTS on brain functions, noxious stimulations were applied to neurons in vitro and in vivo. KBPTS pretreatment in cultured cortical neurons of albino ICR mice rescued death caused by AMPA, NMDA, and kainate as well as by buthionine sulfoximine (BSO) and ferrous chloride (Fe^{2+}) treatments. Furthermore, KBPTS promoted animal's recovery from coma induced by a sublethal dose of KCN and improved survival by a lethal dose of KCN. To examine its physiological effects on the nervous system, we induced ischemia in the Sprague -Dawley rat's brain by middle cerebral artery (MCA) occlusion. Neurological examination showed that KBPTS reduced the time which is required for the animal after MCA occlusion to respond in terms of forelimb and hindlimb movements. Histological examination revealed that KBPTS reduced ischemic area and edema rate and also protected neurons in the cerebral cortex and hippocampus from ischemic damage. Thus, the present data suggest that KBPTS may play an important role in protecting neuronal cells from external noxious stimulations

Key words : Kami-bang-pung-tong-sung-san, neuron, neurotoxicity, middle cerebral artery (MCA) occlusion, hippocampus, cerebral cortex, rat

Introduction

Bang-pung-tong-sung-san(BPTS), which has its original description in Sun-myung-non-bang, a classical treatise of the oriental medicine, is known to be effective in lowering blood viscosity, alleviating inflammatory and allergic reactions, promoting blood circulation, and regulating the autonomic nervous system. BPTS has been used for the treatments of stroke, hypertension, arteriosclerosis, constipation, and cutaneous diseases. Studies using experimental animals have further documented that physiological effects of BPTS are related to anti-analgesic immune responses[25)], anti-allergic, anti-inflammatory, and anti-pyrogenic functions. Similarly, the effects of BPTS on obesity or on the hypertension and hyperlipidemia were reported[14)].

When the brain tissues are exposed to exogenous insults such as ischemic injury and hemorrhage, some brain tissues or neuronal cell types are more susceptible to ischemic injury than others[12,44)]. 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 than others[22,28,36)]. Although molecular mechanism is not completely understood, changes in the levels and metabolism of certain neurotransmitters 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[6,26)]. Oxygen depletion can cause loss of active pumps in neurons, resulting in depolarization of neurons followed by excitotoxic damage of postsynaptic neurons[25,48)]. Deleterious effects by excitatory amino acids (e.g., glutamate in the central nervous system) include cytotoxicity by prolonged and elevated levels of calcium, free radicals, and by activation of molecular machineries of apoptotic pathway[33)]. Indeed, treatment of glutamate or its analogues kainate and NMDA can induce

* To whom correspondence should be addressed at : Dong-Hee Kim, Department of Pathology, College of Oriental Medicine, Youngun-dong, Dong-gu, Daejeon, 300-716 Korea

· E-mail : dhkim@dju.ac.kr, · Tel : 042-280-2623

· Received : 2003/11/28 · Revised :2003/01/09 · Accepted : 2004/01/26

death of cultured primary neurons. Similarly, these agents, when introduced into the brain, cause death of neuronal cell, and also result in pathophysiological abnormalities such as stroke, or epilepsy[15,46,41]. Since hypoxic-ischemic excephalopathy, along with intracranial hemorrhage, is one of 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 aspect, several caspases which are activated during apoptotic cell death is the promising candidate to regulate cell death[26,20,31].

Kami-bang-pung-tong-sung-san (KBPTS) is the modified prescription of BTPS in which Spatholobi Clulis and Salviae Miltiorrzae Radix were added in order to eliminate congestive morphology in diverse tissues caused by injuries. Here, using in vitro and in vivo experimental animal models, we examined whether KBPTS has protective effects from exogenous insults on the brain tissues. Possible neuroprotective effects of KBPTS on neuronal cells were examined in cultured cortical neurons given to cytotoxic insults and also in hippocampal and cortical tissues of rat brain after middle cerebral artery(MCA) occlusion. Our data showed that KBPTS reduced cytotoxic death of cultured cortical neurons induced by AMPA, NMDA or kainate. It was also found that KBPTS has a protective role in brain tissues from ischemic insults by MCA occlusion. Therefore, the present findings suggest that KBPTS may play an important role in protecting neuronal cells from diverse, external insults.

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 protein 21%, crude fat 8%, crude carbohydrate 5%, mineral 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℃, a 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 increases were used for the experiment.

2) Drugs

KBPTS used in the present study was obtained from Daejeon University Oriental Medicine Hospital. One seal of KBPTS has the composition as shown in the Table 1. Augmented KBPTS was prepared by adding 4 g of Spatholobi caulis (Gye-Hyul-Deung) and Salviae miltirrhizae radix (Dan-Sam) each. These two components are recognized to remove effectively congestive damages caused by various insults.

Ten seals of KBPTS were suspended in 2 liters 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. The powder was diluted with physiological saline and used for the present experiment.

Table 1. The Compositions of KBPTS Extracts

Conventional name	Herb name	amount (g)
Cnidii Rhizoma	川芎	4.0
Ledebouriellae Radix	防風	4.0
Angelicae gigantis Radix	當歸	4.0
Paeonia Radix Alba	芍藥	4.0
Menthae Herba	薄荷	4.0
Forsythiae fructuse	連翹	4.0
Ephedrae herba	麻黃	4.0
Natrii sulfas	芒硝	4.0
Rhei Radix ET Rhisoma	大黃	4.0
Gypsum Fibrosum	石膏	4.0
Platycodi Radix	桔梗	4.0
Scutellariae Radix	黃芩	4.0
Atractylodis Macrocephalae Rhizoma	白朮	3.0
Nepetae Herba	荊芥	3.0
Talcum	滑石	10.0
Gardeniae Fructuse	山梔子	3.0
Glycyrrhizae Radix	甘草	2.0
Spatholobi Clulis	鷄血藤	6.0
Salviae Miltiorrzae Radix	丹參	6.0
Total Amount		81

3) Chemicals and Instruments

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

Experimental instruments and apparatus used in this study were as follows; 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), ACL-100 (Instrumentation Laboratory, U.S.A.), physiograph Model 7 (GRASS Instrument Co., Quincy, Mass., U.S.A.), optical microscope (Olympus BH-2., Japan).

2. Methods

1) In vitro cytotoxicity test

(1) Primary cortical neuron culture

Fetal cortices were dissected from mouse embryo (embryonic day 15). Tissues were dissociated, and 1 x 10^5 cells/well were plated on a 24 well plate. The medium used was Eagle's minimal essential media supplemented with 5% horse serum, 5% fetal bovine serum, 2 mM glutamine, and 21 mM glucose. The culture plates were precoated with 100 $\mu g/m\ell$ poly-D-lysine and 4 $\mu g/m\ell$ laminin. Cells were maintained in a CO_2 incubator at 37℃, and ara C was added 3 - 5 days after plating and used for experiment on days in vitro (DIV) 13 - 15.

(2) 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 treating cells with ferrous chloride (Fe^{2+}; 100 μM) or buthionine sulfoximine (BSO; 10 mM). Cells were then 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 release assay.

2) In vivo toxicity test

(1) Induction of global ischemia

Global ischemic model was essentially followed by Schubert method. KBPTS (358 mg/kg body weight) was given to ICR mice by oral injection once a day. Control group animals were administered with an equivalent volume of saline vehicle. Thirty min later, sublethal dose of KCN (1.87 mg/kg) was injected into the arteriole, and the time period until the animals regained the normal orientation reflex was measured. Also, number of animals survived after the injection of lethal dose of KCN (3.0 mg/kg) was determined.

(2) Middle cerebral artery (MCA) occlusion

Two hours before the experiment, Sprague-Dawley (SD) rats were weighed, injected with 2 ml of KBPTS (358 mg/kg body weight) or the same volume of saline vehicle. Animals were subject to MCA occlusion experiment by using the Oral zonda (Daejong Instruments, Korea). Silicon rubber was heated to make 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, rats 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 performed as follows. The central area of the neck was incised, and the right total veins, internal veins, and external veins were exposed. After ligating total veins and external veins, 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. Animals were sacrificed 24 hr later to prepare the brain sections. 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℃. 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.

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

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

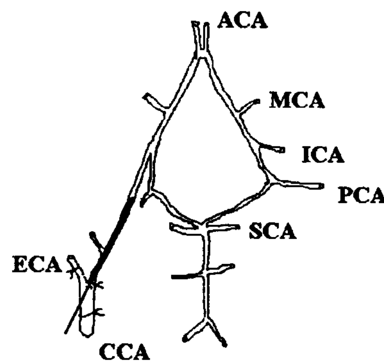

Fig. 1. Schematic representation of insertional positions of silicone rubber cylinder at the right middle cerebral artery (MCA). Diagram also indicates the ligation positions at the ECA (external carotid artery) and CCA; common carotid artery. PCA; posterior cerebral artery, ICA; internal carotid artery, ACA (anterior cerebral artery), SCA, superior cerebral artery.

(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 to 4, and hindlimb was graded 1 and 2.

Table 2. The Neurological Examination Grading System

	Grade	Neurological examination
Forelimb	Grade 1	No deficit
	Grade 2	Forelimb flexation when suspended by the tail
	Grade 3	Reduced forepaw resistance to lateral push
	Grade 4	Circulating behaviour during suspension (body twisting)
Hindlimb	Grade 1	Immediate placement of the behind back on to the table (normal)
	Grade 2	No limb placement / movement

(4) 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, dehydrated with increasing concentrations of ethanol, histocleared, and used for preparing for paraffin blocks. The extent of neuronal damage was determined as follows. The paraffin blocks were cut into 4 μm sections, followed by hydration and cresyl violet staining. After coverslipping, sections were observed under microscope (Olympus-BH2, Japan).

3) Statistical analysis

Number data among groups were compared by unpaired Student's 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. Determination of neuroprotection of KBPTS from cytotoxic stimulation; In vitro study

1) Neuroprotection from excitotoxic amino acids

In order to examine the neuroprotective effects of KBPTS from excitotoxic insults, primary cortical neurons were prepared from brain tissues. Cells were cultured for two weeks and cell death was determined by LDH release assay after the treatment of glutamate receptor agonists. Prolonged stimulation of CNS neurons with glutamate receptor agonists such as NMDA, AMPA, and kainate causes hyperexcitability leading to cytotoxic insults. In our experimental condition, optimal doses for inducing cell death were 20 μg/ml, 20 μg/ml and 50 μg/ml for NMDA, AMPA, and kainate respectively. NMDA treatment (20 μg/ml) alone resulted in LDH release by 90.70±2.19(%) compared to completely dead cells. Addition of KBPTS (10 - 200 μg/ml) slightly reduced levels of cell death (Fig 2). AMPA at 20 μg/ml caused much lower levels of LDH release (48.27±2.60%) compared to NMDA treatment. Then, the addition of KBPTS (10 - 200 μg/ml) decreased LDH release lower than 25% (Fig 2). Finally, neuroprotective effects of KBPTS were similarly observed in kainate treated cells. Kainate alone (50 μM) induced LDH release (43.49±3.71%), whereas LDH release was reduced lower than 21% in cells treated with KBPTS at 10 - 200 μg/ml (Fig 2). Together, these data suggest that KBPTS was effective in protecting cortical neurons treated with AMPA or kainate.

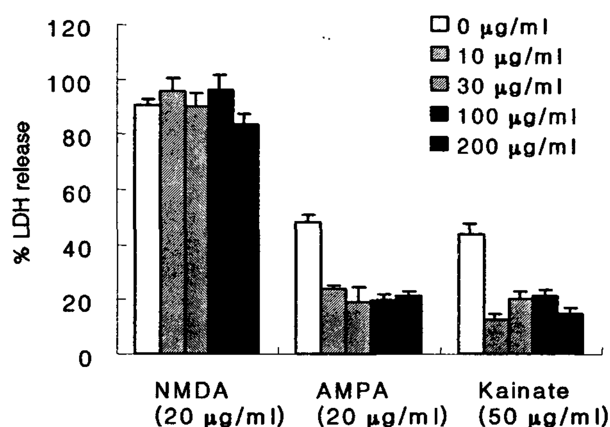

Fig. 2. Inhibitory effects of KBPTS on cultured cortical neuronal death mediated by glutamate receptor agonists. Levels of cytotoxicity were determined by LDH release. The Glutamate agonists NMDA (20 μM), AMPA (20 μM) and kainate (50 μM) were treated to cells for 24 hr. Figure shows that KBPTS at higher than 10 μg/ml significantly reduced levels of cell death by AMPA and kainate treatments.

2) Cell protection from free radicals

Primary cortical neurons on DIV 13-15 were treated with 10 mM BSO or equivalent volume of vehicle. Levels of LDH release were 45.74±0.64(%) in saline vehicle control group, and then levels of LDH were decreased to 17.34±4.94(%), 6.02±1.63(%) for 100 and 300 μg/mℓ of KBPTS concentrations respectively. Further increases in KBPTS concentration to 1000 or 2000 μg/mℓ resulted in slight increases in cell death (; 14.63±4.07(%), 32.50±4.23(%) respectively).

In cells treated with 100 μM Fe^{2+}, LDH release levels were 51.99±4.49(%). Then the addition of KBPTS (100 - 2000 μg/ml) decreased levels of LDH release in a dose-dependent manner (% levels of LDH release; 20.06±1.71(%), 9.41±2.33(%), 8.01±1.00(%), and 7.34±0.30(%) for 100, 300, 1000, and 2000 μg/mℓ of KBPTS treatments respectively) (Fig 3).

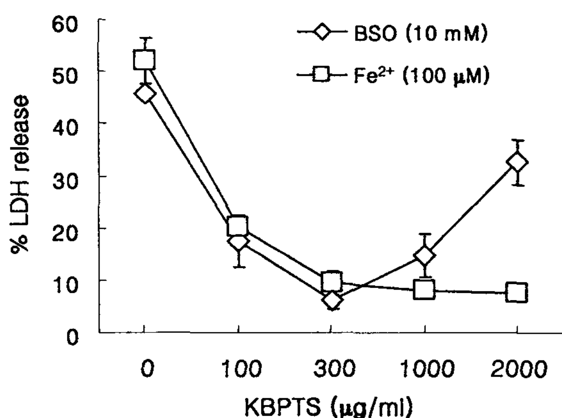

Fig. 3. Inhibitory effect of KBPTS on LDH release induced by BSO and Fe2+ in cultured cortical neurons

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

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

In KCN-treated ICR mice, the extent of animal's unconsciousness was first determined. Coma duration after treatment with a sublethal dose of KCN (1.87 ㎎/kg i.v) was 20.57±4.45 sec. In mice treated with KBPTS-treated animals, the duration was shortened (19.84±5.66 sec), but not statistically significant (Fig 4). With a lethal dose of KCN treatment (3.0 ㎎/kg i.v.), the survival rate of KBPTS group was 20% (; one of 10 animals examined survived) at which dose the saline injected control animals (n = 10) were all dead.

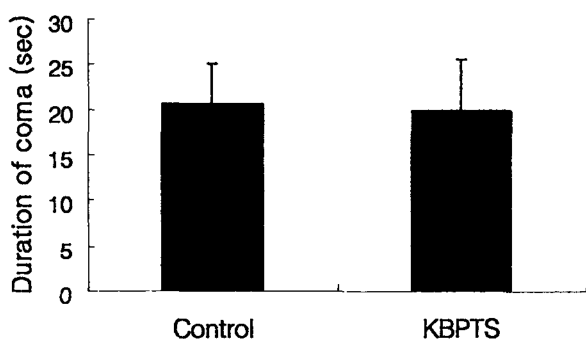

Fig 4. The duration of coma in albino ICR mice by KCN. KCN (1.87 ㎎/kg) were intravenously injected after oral administration of 460 mg/kg of KBPTS extracts (KBPTS) or the equivalent volume of normal saline (Control). The error bars represent the standard error of mean (SEM). n (number of animals) = 10 for each group.

2) MCA occlusion tests

Ischemic area and edema rate by MCA occlusion were examined in Sprague-Dawley rats. Ischemic ratio was determined in KBPTS-treated and saline-treated animals (n = 4 each group). The extent of ischemic damage was compared at 8 different levels of coronal brain slices spanning whole brain area between two groups. As shown in Table 3, the mean area of infarction was observed in 7 of 8 slices in saline-treated animals showing values ranging 4 - 20% whereas no infarction was detected in KBPTS-treated animals. A representative example of coronal brain sections was shown in Fig 5, indicating no appearance of ischemic area in KBPTS-treated animal's brain. We further investigated the protective effects of KBPTS on ischemic insults by measuring the edema ratio. As shown in Table 4, the edema rate was significantly reduced in KBPTS-treated animals compared to saline control. Only 3 out of 8 slices examined showed 1 - 4% of edema while 6 out of 8 slices in saline control showed edema rate up to 21%.

Table 3. Ischemic ratio in MCA-occluded Sprague-Dawley Rats

Serial numbers of slices examined	*Mean area of infarction (%)	
	Control	KBPTS
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

* mean ± SEM, n (number of animal) = 4 for each group.
Control : Oral administration of physiological saline
KBPTS : Oral administration of KBPTS (462.5㎎/kg)

Table 4. Edema ratio in MCA-occluded Sprague-Dawley rats

Serial numbers of slices examined	*Edema rate (%)	
	Control	KBPTS
1	11.71 ± 3.56	1.15 ±4.67
2	20.87 ± 5.28	2.27 ± 3.38
3	17.03 ± 3.26	3.90 ± 109
4	19.15 ± 4.79	0
5	7.35 ± 1.89	0
6	1.17 ± 3.86	0
7	0	0
8	0	0

* mean ± SEM, n (number of animal) = 4 for each group.
Control : Oral administration of physiological saline
KBPTS : Oral administration of KBPTS (462.5㎎/kg)

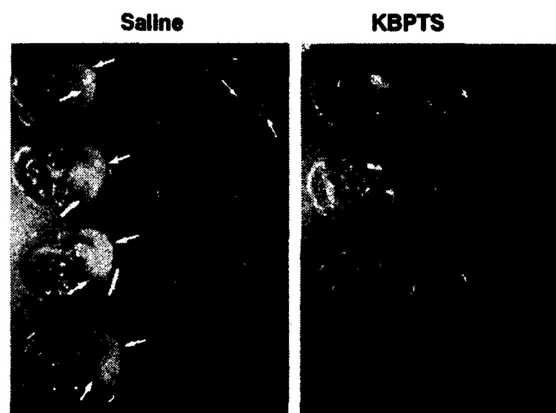

Fig. 5. Pattern of ischemia in MCA-occluded Sprague-Dawley rats. In saline treated group, the areas pointed by arrows indicate the brain regions of ischemic damage. In contrast, animals treated KBPTS (786 mg/kg) showed no evident ischemic damage in coronal sections of brain tissues examined.

3) Neurological examination

To investigate the effects of KBPTS on behavioral recovery after ischemic injury, forelimb and hindlimb movements were measured in animals after MCA occlusion. In forelimb test, scores of movement behavior were graded 1 - 4 as shown in Table 2; Note that the scores of lower number indicate the movement behavior close to normal animal. The scores through the operation, hypoxia and recirculation period were higher in the control group than KBPTS-treated animals (Table 5). Similarly in the hindlimb test where the score was graded 1 or 2, recovery behavior of KBPTS-treated groups were clearly improved compared to saline control animals.

Table 5. Behavioral effects of KBPTS treatment in Sprague-Dawley Rats

Sample		Operation	Hypoxia	Recirculation
Forelimb	Control	3.00±0.00	2.25±0.25	2.5±0.28
	KBPTS	1.5±0.28	1.5±0.25	1.75±0.25
Hindlimb	Control	1.00±0.00	1.00±0.00	1.00±0.00
	KBPTS	0.5±0.28	0.75±0.25	0.5±0.28

Number data in the Table represent mean score ± SEM (n = 4).
* mean ± SEM, n (number of animal) = 4 for each group.
Control : Oral administration of physiological saline
KBPTS : Oral administration of KBPTS (283 ㎎/kg)

4) Histological examinations on the effects of KBPTS treatment on cortical and hippocampal tissues after MCA occlusion

In normal animals, the six-layered organization of cortical structures was clearly observed. Particularly strong cell body staining was seen in the large pyramidal cells in layers 3 and 5 (Fig. 6A). In animals with the MCA occlusion, layered structures were not clear, nuclei were shrunken, the border between cells was unclear, and various sizes of vacuoles were observed (Fig. 6B). In KBPTS-treated animals, although the staining pattern of cortical cells in the hemisphere with ischemic insults was more irregular compared to uninjured control, staining integrity was essentially maintained (Fig. 6C).

Hippocampus is one of the brain areas which is sensitive to various kinds of external insults including ischemic damage. In an untreated animal group, strong staining was observed in the pyramidal-shape cells and weak staining in round-shape cells in the hippocampal pyramidal cells and dentate gyrus granule cells (Fig. 6D). Then, MCA occlusion generated the signs of ischemic damages manifested by unclear cell morphologies in the hippocampal formation as well as granule cells. Also, numerous vacuoles were observed. (Fig. 6E). In KBPTS group, the number of hippocampal pyramidal, but not granule, cells were close to that of uninjured control and morphologies of pyramidal cells and granule cells were much more intact compared to tissues from MCA occlusion (Fig. 6F).

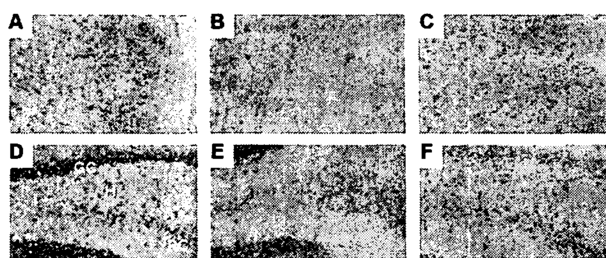

Fig. 6. Protection of KBPTS of cortical and hippocampal tissues from ischemic insults by MCA occlusion. MCA occlusion and treatment of KBPTS were performed in the Sprague-Dawley rats as described in Materials and Methods. Coronal brain sections were stained with cresyl fast violet stain, and photographed (X100). A - C: cerebral cortex, D - F: hippocampus. A and D: intact tissue, B and E: tissues with MCA occlusion, C and F: tissues with MCA occlusion plus KBPTS treatment. GC: granule cells, H: hilus, PC: pyramidal cells.

Discussion

KBPTS has been widely used in the oriental medicine for the treatment of several diseases associated with inflammation in cardiovascular and nervous systems. In the present study, we found that KBPTS protects neuronal cells from excytotoxic insults in vivo as well as in vitro by lowering the cell's responsiveness to noxious stimulations such as excitotoxic amino acids or free radicals in neuronal cells. KBPTS rescued cultured cortical neurons from death induced by AMPA, NMDA and kainate as well as buthionine sulfoximine (BSO) and ferrous chloride (Fe^{2+}) treatments. Also, KBPTS promoted animals to recover from coma induced by a sublethal dose of KCN, and increased survival rate by lethal dose of KCN. After MCA occlusion, KBPTS appeared to protect brain tissues such as cortical and hippocampal tissues from ischemic injury. Thus, the present data suggest that KBPTS may play an important role in protecting neuronal cells from external noxious stimulations.

KBPTS is a mixture prescription containing more than 10 kinds of herbs, and it is not surprising to predict that its effects on regulating cell fate would be complicated, as would be case for other herb prescriptions. Our experimental approach was therefore to examine its effect in different physiological systems including cardiovascular system and nervous systems. In the accompanying paper, we found that in the spontaneously hypertensive rat (SHR) administered with KBPTS, blood pressure and heartbeat rate were significantly reduced compared to non-treated SHR (Na et al., in this issue). In the present study, protective effects of KBPTS on the nervous system were investigated using both in vitro culture system and in vivo experimental animal models. Excitatory neurotransmitter glutamate analogues resulted high levels of neuronal death, but cells pretreated with KBPTS were significantly protected from excitotoxic death. KBPTS, when

treated with AMPA or kainate rescued cells from death measured by LDH release. High doses of NMDA, AMPA, and kainate are known to cause cell damage by protracting hyperexcitability in postsynaptic neurons[27,29,37,42,48]. Prolonged increases in cytoplasmic calcium[30], reactive oxygen species, and others[38] are believed to cause cell damage. Despite the differences in cytotoxicity by NMDA, AMPA, and kainate, several lines of evidences suggested increased production of free radicals in the cytoplasm as a common denominator causing cell damage. Consistent with this possibility is our data which showed KBPTS-mediated neuroprotection from BSO or Fe^{2+}-treated cortical neurons. BSO and Fe^{2+} are known to increase the production of free radicals[40,49,17,19,23]. Further studies to identify chemical compositions of KBPTS would be critical to understand molecular mechanisms underlying KBPTS-mediated neuronal protection.

The protective effect of KBPTS was also demonstrated using in vivo ischemic injury model. As an experimental model of global ischemia, neurotoxic agent KCN[21] was injected into rats which were pretreated with KBPTS 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[3,4,5,10,11]. Our study shows that KBPTS treatment with lethal dose improved animal's survival. Moreover, when sublethal dose of KCN was given, the recovery time from unconsciousness was faster in KBPTS-treated animals than control animals. These data suggest that KBPTS 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 KBPTS treatment protected hippocampal pyramidal neurons and cerebral pyramidal neurons from ischemic damage caused by the MCA occlusion. The MCA occlusion is the widely accepted, experimental model of ischemic injury[16,18,34,24] and neuronal cells are one of the most susceptible targets of ischemic injury[35,39,9]. 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 the 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[25]. While neuronal damage causes cell death primarily by necrosis, recent studies indicate that ischemic insults can activate caspase-activated apoptotic pathway[13,31,43]. We found that in KBPTS-treated animals, mean ischemic ratio and the extent of edema were significantly reduced after ischemic injury. Also, KBPTS 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 studies on KBPTS in relation to animal's behavioral activity will be of particular interest.

Conclusion

By using in vitro and in vivo approaches, we studied the potential protective role of KBPTS in the nervous system. KBPTS appeared to protect neuronal cells from various external stimulations. Further studies are required to examine molecular mechanism on which molecular components act on reducing cytotoxicity. To do this, it would be critical to analyze the chemical compositions of KBPTS. It should be also kept in mind that some components of KBPTS might have adverse effects which should be carefully sorted out and eliminated from the prescription for the purpose of basic research as well as clinical applications.

References

1. Aihara N, Tanno H, Hall JJ, Pitts LH, Noble LJ. : Immunocytochemical localization of immunoglobulins in the rat brain: relationship to the blood-brain barrier. J Comp Neurol. 342(4):481-96, 1994.
2. Albayrak S, Zhao Q, Siesjo BK, Smith ML. : Effect of transient focal ischemia on blood-brain barrier permeability in the rat: correlation to cell injury. Acta Neuropathol. 94(2):158-63, 1997.
3. Barone FC, Price WJ, White RF, Willette RN, Feuerstein GZ. : Genetic hypertension and increased susceptibility to cerebral ischemia. Neurosci Biobehav Rev 16:219-233, 1992.
4. Barone FC, Schmidt DB, Hillegass LM, Price WJ, White RF, Feuerstein GZ, Clark RK, Griswold DE, Sarau HM. : Reperfusion increases neutrophil and LTB4 receptor binding in focal ischemia. Stroke 23:1337-1348, 1992.
5. Barone FC, Clark RK, Price WJ, White RF, Storer BL, Feuerstein GZ, Ohlstein EH. : Neuron specific enolase

increases in cerebral and systemic circulation following focal ischemia. Brain Res 623:77-82, 1993.

6. Baylor AE 3rd, Diebel LN, Liberati DM, Dulchavsky SA, Brown WJ, Diglio CA. : The synergistic effects of hypoxia/reoxygenation or tissue acidosis and bacteria on intestinal epithelial cell apoptosis. J Trauma. 55(2):241-7; discussion 247-8, 2003.
7. Binaschi A, Bregola G, Simonato M. : On the role of somatostatin in seizure control: clues from the hippocampus. Rev Neurosci. 14(3):285-301, 2003.
8. Bliss TV, Collingridge GL. : A synaptic model of memory: long-term potentiation in the hippocampus. Nature. 361(6407):31-9, 1993.
9. Borlongan CV, Yamamoto M, Takei N, Kumazaki M, Ungsuparkorn C, Hida H, Sanberg PR, Nishino H. : Glial cell survival is enhanced during melatonin-induced neuroprotection against cerebral ischemia. FASEB J. 14(10):1307-17, 2000.
10. Brint S, Jacewicz M, Kiessling M, Tanable J, Pulsinelli W. : Focal brain ischemia in the rat: methods for reproducible neocortical infarction using tandem occlusion of the distal middle cerebral artery and ipsilateral common carotid arteries. J Cereb Blood Flow Metab 8:474-485, 1988.
11. Buchan AM, Xue D, Slivka A. : A new model of temporary focal neocortical ischemia in the rat. Stroke 23:273-279, 1992.
12. Cantagrel S, Krier C, Ducrocq S, Bodard S, Payen V, Laugier J, Guilloteau D, Chalon S. : Hypoxic preconditioning reduces apoptosis in a rat model of immature brain hypoxia-ischaemia. Neurosci Lett. 347(2):106-10, 2003.
13. Clark RK, Lee EV, Fish CJ, White RF, Price WJ, Jonak ZL, Feuerstein GZ, Barone FC. : Progression of cerebral changes following middle cerebral artery occlusion in the rat: a quantitative planimetric, histologic, and immunohisto-chemical study. Brain Res Bull 31:565-572, 1993.
14. Lee, YH : Effect of Bangpoongtongsungsan on the Hypertention induced by L-NAME injection and SHR in Rats), Dongeui University, Graduate School thesis, 1999.
15. During MJ, Symes CW, Lawlor PA, Lin J, Dunning J, Fitzsimons HL, Poulsen D, Leone P, Xu R, Dicker BL, Lipski J, Young D. : An oral vaccine against NMDAR1 with efficacy in experimental stroke and epilepsy. Science. 287(5457):1453-60, 2000.
16. Duverger D, MacKenzie T. : The quantification of cerebral infarction following focal ischemia in the rat: influence of strain, arterial pressure, blood glucose concentration, age. J Cereb Blood Flow Metab 8:449-461, 1988.
17. Grasbon-Frodl EM, Andersson A, Brundin P. : Lazaroid treatment prevents death of cultured rat embryonic mesencephalic neurons following glutathione depletion. J Neurochem. 67(4):1653-60, 1996.
18. Hara H, Ayata C, Huang PL, Waeber C, Ayata G, Fujii M, Moskowitz MA. : [3H]L-NG-nitroarginine binding after transient focal ischemia and NMDA-induced excitotoxicity in type I and type III nitric oxide synthase null mice. J Cereb Blood Flow Metab. 17(5):515-26, 1997.
19. Jellinger KA. : General aspects of neurodegeneration. J Neural Transm Suppl. 65:101-44, 2003.
20. Jeremias I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P, Debatin KM. : Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia. Circulation. 102(8):915-20, 2000.
21. Jones DC, Gunasekar PG, Borowitz JL, Isom GE. : Dopamine-induced apoptosis is mediated by oxidative stress and Is enhanced by cyanide in differentiated PC12 cells. J Neurochem. 74(6):2296-304, 2000.
22. Kokaia Z, Lindvall O. : Neurogenesis after ischaemic brain insults. Curr Opin Neurobiol. 13(1):127-32, 2003.
23. Lan J, Jiang DH. : Excessive iron accumulation in the brain: a possible potential risk of neurodegeneration in Parkinson's disease. J Neural Transm. 104(6-7):649-60, 1997.
24. Le DA, Wu Y, Huang Z, Matsushita K, Plesnila N, Augustinack JC, Hyman BT, Yuan J, Kuida K, Flavell RA, Moskowitz MA. : Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. Proc Natl Acad Sci U S A. 99(23):15188-93, 2002.
25. Lee JM, Zipfel GJ, Choi DW. : The changing landscape of ischaemic brain injury mechanisms. Nature. 399(6738 Suppl):7-14, 1999.
26. Manabat C, Han BH, Wendland M, Derugin N, Fox CK, Choi J, Holtzman DM, Ferriero DM, Vexler ZS. : Reperfusion differentially induces caspase-3 activation in ischemic core and penumbra after stroke in immature brain. Stroke. 34(1):207-13, 2003.
27. McCulloch J. : Glutamate receptor antagonists in cerebral ischaemia. J Neural Transm Suppl. 43:71-9, 1994.
28. Meldrum BS. : Concept of activity-induced cell death in epilepsy: historical and contemporary perspectives. Prog Brain Res. 135:3-11, 2002.
29. Miyazaki S, Katayama Y, Furuichi M, Kano T, Yoshino A, Tsubokawa T. : N-methyl-D-aspartate receptor-mediated, prolonged afterdischarges of CA1 pyramidal cells following transient cerebral ischemia in the rat hippocampus in vivo. Brain Res. 657(1-2):325-9, 1994.
30. Namgung U, Kim DH, Lim SR, Xia Z. : Blockade of

calcium entry accelerates arsenite-mediated apoptosis in rat cerebellar granule cells. Mol Cells. 30;15(2):256-61, 2003.

31. Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA. : Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci. 18(10):3659-68, 1998.
32. Nicoll RA, Malenka RC. : Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature. 377(6545):115-8, 1995.
33. Offen D, Elkon H, Melamed E. : Apoptosis as a general cell death pathway in neurodegenerative diseases. J Neural Transm Suppl. 58:153-66, 2000.
34. Okada M, Nakanishi H, Tamura A, Urae A, Mine K, Yamamoto K, Fujiwara M. : Long-term spatial cognitive impairment after middle cerebral artery occlusion in rats: no involvement of the hippocampus. J Cereb Blood Flow Metab. 15(6):1012-21, 1995.
35. Philip K. Liu, Robert G. Grossman, Chung Y. Hsu and Claudia S. Robertson : Ischemic injury and faulty gene transcripts in the brain, Trends in Neurosciences, 24(10):581-588, 2001.
36. Rami A. : Ischemic neuronal death in the rat hippocampus: the calpain-calpastatin-caspase hypothesis. Neurobiol Dis. 13(2):75-88, 2003.
37. Rajdev S, Reynolds IJ. : Glutamate-induced intracellular calcium changes and neurotoxicity in cortical neurons in vitro: effect of chemical ischemia. Neuroscience. 62(3):667-79, 1994.
38. Reynolds IJ, Hastings TG. : Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. J Neurosci. 15(5 Pt 1):3318-27, 1995.
39. Robertson SC, Loftus CM. : Effect of N-methyl-D-aspartate and inhibition of neuronal nitric oxide on collateral cerebral blood flow after middle cerebral artery occlusion. Neurosurgery. 42(1):117-23, 1998.
40. Seo SY, Kim EY, Kim H, Gwag BJ. : Neuroprotective effect of high glucose against NMDA, free radical, and oxygen-glucose deprivation through enhanced mitochondrial potentials. J Neurosci. 19(20):8849-55, 1999.
41. Shoham S, Youdim MB. : Iron involvement in neural damage and microgliosis in models of neurodegenerative diseases. Cell Mol Biol (Noisy-le-grand). 46(4):743-60, 2000.
42. So Y. Seo, Eun Y. Kim, Harriet Kim, and Byoung J. Gwag : Neuroprotective Effect of High Glucose Against NMDA, Free Radical, and Oxygen-Glucose Deprivation through Enhanced Mitochondrial Potentials. J. Neurosci., 19: 8849-8855, 1999.
43. States BA, Honkaniemi J, Weinstein PR, Sharp FR. : DNA fragmentation and HSP70 protein induction in hippocampus and cortex occurs in separate neurons following permanent middle cerebral artery occlusions. J Cereb Blood Flow Metab. 16(6):1165-75, 1996.
44. Tian H, Zhang Q, Li H, Zhang G. : Antioxidant N-acetylcysteine and AMPA/KA receptor antagonist DNQX inhibited mixed lineage kinase-3 activation following cerebral ischemia in rat hippocampus. Neurosci Res. 47(1):47-53, 2003.
45. Whitney KD, McNamara JO. : Autoimmunity and neurological disease: antibody modulation of synaptic transmission. Annu Rev Neurosci. 22:175-95, 1999.
46. Winkler DT, Scotti AL, Nitsch C. : Ischemia-induced degeneration of CA1 pyramidal cells decreases seizure severity in a subgroup of epileptic gerbils and affects parvalbumin immunoreactivity of CA1 interneurons. Exp Neurol. 168(2):364-72, 2001.
47. Yang R, Puranam RS, Butler LS, Qian WH, He XP, Moyer MB, Blackburn K, Andrews PI, McNamara JO. : Autoimmunity to munc-18 in Rasmussen's encephalitis. Neuron. 28(2):375-83, 2000.
48. Ying HS, Weishaupt JH, Grabb M, Canzoniero LM, Sensi SL, Sheline CT, Monyer H, Choi DW. : Sublethal oxygen-glucose deprivation alters hippocampal neuronal AMPA receptor expression and vulnerability to kainate-induced death. J Neurosci. 17(24):9536-44, 1997.
49. Zeevalk GD, Bernard LP, Sinha C, Ehrhart J, Nicklas WJ. : Excitotoxicity and oxidative stress during inhibition of energy metabolism. Dev Neurosci. 20(4-5):444-53, 1998.