

# **THE HERBAL PRESCRIPTION YOUKONGDAN MODULATES RODENT MEMORY, ISCHEMIC DAMAGE AND CORTICAL mRNA GENE EXPRESSION**

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*The effects of the herbal prescription youkongdan (YKD) on memory performance of rodents following cerebral ischemia/reperfusion and scopolamine administration were measured and compared to the age-related changes in mRNA expression induced by dietary supplementation of YKD. Following ischemia, YKD decreased neuronal cell loss in the CA1 region of rat hippocampus by 89% relative to controls. YKD improved the water maze performance of both ischemic and scopolamine-treated animals. Dietary administration of YKD resulted in significant modulation of Egr1, Grp78, Hsp86, SOD1, and  $\alpha$ B crystallin mRNA expression and a trend toward increased exploratory behavior in older mice.*

**Keywords** aging, brain, ischemia, mRNA, scopolamine, youkongdan

Mitigation of the events underlying functional decline in the normal aging brain is desirable not only in the case of nonpathological senescence but also in the case of a broad range of neurodegenerative diseases. Age-associated neurodegenerative disease is characterized by long-term progression and a multifactorial etiology. Disorders such as stroke and memory loss characteristic of senile dementia and nonfamilial Alzheimer's disease are rare in the young adult but occur with rapidly increasing frequency in later life. Thus the benefits ensuing from slowing the progression of adverse events associated with brain senescence are not confined to normal aging but are likely to impact the age of onset and the incidence of many neurological disorders. An effective approach to delaying their progression may be by chronic consumption of dietary supplements containing multiple active agents. These same multiple agents may also be found effective in the short-term treatment of some neurodegenerative conditions, such as stroke and memory loss.

Changes in immune function and the presence of an imbalance between pro-oxidant and antioxidant factors have both been repeatedly shown to occur with cerebral aging (Hull et al., 1996; Bondy, 1998; Sohal, 1998; Calabrese et al., 2000) and stroke (Kuroda & Siesjo, 1997). These age-related changes are toward increasing levels of oxidative damage to macromolecules together with an impaired immune response. The involvement of such diverse deleterious changes associated with neurodegenerative disease implies that multiple active components, such as those contained in a multi-herbal prescription, may be necessary for effective prevention or treatment.

The purpose of this study was to investigate the mitigation of age-related deficits in CNS function by short-term and dietary administration of the Korean herbal prescription *youkongdan* (YKD; Table 1), as indicated by normalization of brain histology and of expression levels of stress-related genes, and as demonstrated by improved learning behavior in YKD-treated animals. Low toxicity and numerous beneficial effects have been noted for the individual herbal ingredients of YKD, so there is the expectation that these benefits may be enhanced by their combination.

## MATERIALS AND METHODS

### YKD Extract Solution

A lyophilized aqueous extract of the *youkongdan* herbal prescription (YKD extract, Jaseng Hospital, Seoul, Korea) was dissolved in normal saline to produce a concentration of 1000 mg/ml.

### YKD Dietary Supplementation

Female B6D2F1 mice, a hybrid between the C57BL/6 and DBA/2 strains from Harlan Labs (Indianapolis, IN, USA), aged 5 months (young group), 17 months (middle-aged group), and 28 months (old group), were housed 4 per cage and were maintained on a 12-h light/dark cycle in a temperature controlled ( $22 \pm 1^\circ\text{C}$ ) room. Food and water were provided ad libitum to animals in each group for 13 weeks. The pelleted minimal AIN93M basal diet (#110900, Dyets Inc., Bethlehem, PA, USA) consisted of 10% sucrose, 47% corn starch, 14% casein, 4% soybean oil, and 21% fiber (w/w) as well as a minimal salt and vitamin mix. For one group of mice of each age this was supplemented with 1.0% (w/w) YKD (a gift from Jaseng Hospital, Seoul, Korea).

### Global Cerebral Ischemia by 4-Vessel Occlusion (4-VO)

Male Wistar rats (SPF, Slc, Japan) aged 6 weeks and weighing between 160 and 180 g were used for both ischemia and scopola-

TABLE 1. Composition and properties of the Korean herbal prescription *youkongdan*

Ingredient	Species	Weight Percent	Indications	Active Components	References
Astragali radix, Huang se 黃芪	<i>Astragalus membranaceus</i>	13.2%	Common cold, wound ulcers, nephritis	Astragalosides I, II, and III	Duke, Huang
Atractylodis rhizoma alba 蒼朮	<i>Atractylodes japonica</i>	8.8%			Duke
Angelicae gigantis radix 當歸	<i>Angelica gigas</i>	8.8%		Decursin	Duke
Longanae arillus 龍眼肉	<i>Euphoria longana</i>	8.8%	Antifungal, anxiolytic	Adenine, choline, glucose, adenosine	Bensky, Okuyama
Lycii folium, Di gu pi 地骨皮	<i>Lycium chinense</i>	8.8%	Diabetes, hypertension, wasting disease	$\beta$ -carotene, ascorbic acid, thiamine, niacin, riboflavin	Duke, Huang
Cnidii rhizoma 川芎	<i>Cnidium officinale</i>	6.7%		Tetramethylpyrazine	Duke
Hoellen, Fu ling 茯苓	<i>Portia cocos</i>	4.4%	Antibacterial, antiviral, hypoglycemic	Pachymic, tumulosic, eburigic and pinicollic acids	Huang
Glycyrrhizae radix, licorice root, Gan cao 甘草	<i>Glycyrrhiza uralensis</i>	4.4%	Antitussive, demulcent, emollient, expectorant, and laxative	Glycyrrhetic acid, glycyrrhizin	Duke, Huang
Carthami flos 紅花	<i>Carthamus tinctorius</i>	1.3%	Amenorrhea		Duke
Cervi parvum cornu, Lu rong 鹿茸	<i>Cervus nippon</i>	4.4%	Growth stimulant, appetite stimulant, improves sleep	Vitamin A, estrone, estranediol	Huang
Crataegi fructus, Shan zha 山楂子	<i>Crataegus pinnatifida</i> var. <i>typica</i>	4.4%	antiscorbutic, deobstruent, laxative, stimulant, stomachic	Chlorogenic, caffeic, citric, crataegolic, maslinic and	Duke, Huang

Rehmanniae radix preparata, Di huang 地黃	Rehmannia glutinosa var. purpurea	4.4%	cardiotonic, diuretic, hemostatic, hypoglycemic; treat diabetes	Catalpol	Duke, Huang
Saussureae radix 木香	Saussurea lappa	4.4%	Phototoxic and weakly antibiotic to <i>Candida albicans</i>	Aplotaxene, camphene, $\alpha$ - costene, $\beta$ -costene, costol, kushitin, phellodrene	Duke
Amomi fructus, Cao guo 草果	Amomum tsao-ko	4.4%	Expectorant, antimalarial		Huang
Polygonum multiflori radix, Shou wu 何首烏	Polygonum multiflorum	4.4%	Laxative, lower cholesterol	Chrysophenol, emotin, rhein, polygonimitin	Duke, Huang
Paeoniae radix alba 芍藥	Paeonia albiflora var. trichocarpa	4.4%		Paeoniflorin	
Ginseng radix 人蔘	Panax ginseng	4.4%	antiseptic, carminative, demulcent, stimulant, tonic	Panaxin, panaxic acid, panaquillon, panacene, gensenhin, ginsenoside	Duke, Huang

mine testing. Before surgery, food was withheld overnight but water was freely available. Rats were anesthetized with 5% isoflurane (Baxter, USA) in a mixture of 70% nitrogen and 30% oxygen; anesthesia was maintained with 1.5% isoflurane. After anesthetization, a rat was laid on its back in a stereotaxic apparatus with the head held at a downward angle of 30° on the horizontal die of the apparatus while the nose and the mouth were fitted into a plastic cone connected to an anesthesia device (Ohmeda V.M.C./Boc Health Care, Cyprian, UK). The tail was fixed on the operating table while the cervical vertebrae were extended. First the throat region was opened and silicon tube rings were inserted into the common carotid arteries. Next, the rat was laid on its stomach and a micro electrocautery needle  $\leq 1$  mm in diameter was inserted through the alar foramina of the first cervical vertebra into the tunnel through which the vertebral artery runs. Both branches of the vertebral artery were occluded by electrical cauterization; operating clips were used as sutures. After 24 h, a thread was passed through the cervical and paravertebral muscles and between the trachea and the esophagus so as to occlude the external jugular vein and the common carotid arteries, after which the wounds were sutured with operating clips. To induce ischemia, the silicon tube rings were tightened so as to occlude the common carotid arteries. If the rat's light reflex vanished within 1 min, the cervical thread was tightened further. After 10 min of occlusion, the tube rings were loosened and the thread was removed, permitting reperfusion. Only those rats that were unconscious for  $20 \pm 5$  min after reperfusion were chosen for further study. Sham animals were operated on similarly, but no fore-brain ischemia was induced.

The body temperature of each rat was monitored every 30 min for 6 h after the induction of ischemia. Heat lamps were used to maintain body temperature at  $37 \pm 5^\circ\text{C}$  during induction of ischemia, reperfusion, and recovery. To estimate brain temperature, body temperature was measured with a probe inserted at least 6 cm into the rectum (Miyazawa & Hossmann, 1992). YKD extract solution (100 mg/kg, i.p.) was administered to rats 0 and 90 min after induction of ischemia. Ischemia-only animals were injected i.p. with 180  $\mu\text{l}$ /kg distilled water at the same time points. Beginning the day after ischemia induction, some animals were administered 200  $\mu\text{l}$  YKD

extract solution p.o. daily for seven days. Animals retained for behavioral testing were administered YKD for an additional seven days during the test period.

### **Scopolamine Treatment**

Male Wistar rats (SPF, Slc, Japan) aged 6 weeks and weighing between 160 and 180 g and female C57BL/6 mice aged 16 weeks were used. Animals were injected with scopolamine (1 mg/kg, i.p., Sigma #s-0929) during water maze testing as described below. Control animals were injected with an equal volume of physiological saline.

### **Water Maze Testing**

Spatial memory tests were performed as described (Morris, 1984) with slight modification. A round pool (for rats, mice: diameter, 186 cm, 100 cm; height, 60 cm, 30 cm), was filled to a height of 10 cm below the tank rim with  $22 \pm 1^{\circ}\text{C}$  water made opaque with powdered milk. The pool was situated in a dimly lit room and surrounded by four uniformly distributed visual cues for orientation. A plastic platform 10 cm in diameter was placed 1 cm below the water surface, midway between the center and rim of the pool in one quadrant. A video tracking system (Noldus, the Netherlands) was used to record animal location and to determine total swim latency (time to reach the platform), distance, and speed. An efficiency ratio was calculated by dividing the animal's swim distance by the straight-line distance from the entry point to the platform.

Animals from the various experimental groups were tested in a random order that was repeated on each day of testing; testing was performed from 10 pm to 3 am, corresponding to the animals' active period. Working memory testing, the acquisition trials, lasted for five consecutive days, with a two-session trial given on each of days 2–5. Day 1 consisted of 90 s swimming in the presence of the platform. Twice on each of the next four days, animals were tested in the presence of the platform in two identical sessions (Sessions 1 and 2), conducted as follows. An animal was placed in the water near the perimeter of one quadrant, but released facing a direction that varied

with each session. Once the animal located and climbed on the platform, it was allowed to rest for 30 s. If, after 90 s, an animal had not located the platform, the experimenter would place it on the platform for 30 s. The second session was begun 20 min after the end of the first. The platform location was changed on each subsequent day. For the probe trial on day 6, the platform was removed and the animal was released as before. The decrease in session 1 escape latency from one day to the next, and the probe trial latency, represent improvement in long-term memory, whereas that from session 1 to session 2 measures improvement in working or short-term memory (Morris, 1983). Prior to the first acquisition trial, animals in the scopolamine group were injected with scopolamine (1 mg/kg, i.p.) or saline. Rats, both ischemia and scopolamine groups, received either YKD extract (100 mg/kg, 200  $\mu$ l, p.o.) or 200  $\mu$ l saline p.o. daily during the test period. For mice in the scopolamine experiment, the YKD dosage was 1000 mg/kg.

### **Measurement of Locomotor Activity**

During the week prior to sacrifice, mice in the dietary study were tested for open field locomotor activity by using a Digiscan Animal Activity Monitor (Accuscan Inc., Columbus, OH, USA). The testing apparatus consisted of an empty plastic cage (40  $\times$  40  $\times$  30 cm) with 16 photocell detectors along two perpendicular sides of the box and 16 light-emitting diodes (LEDs) along the other two sides. Another set of 8 LEDs and detectors were mounted 7.5 cm above the floor to detect vertical rearing activity. The test subjects are unaware of the invisible infrared light beams so that behavior is unaffected by the monitoring instrument. Testing was conducted between 7 am and 5 pm; starting times were randomized among groups. Each animal was tested for 30 min at the same time of day on each of two successive days; the sum of the two values for each parameter was used. The apparatus was cleansed with detergent between trials to remove residual odors.

Total distance traveled was measured as an indicator of ambulatory activity. Vertical rearing behavior was measured as an indicator of a mouse's ability to alter its perspective to gain increased knowledge of its surroundings. The difference between the time



spent at cage margins and that spent in the central area of the test chamber was measured and is interpreted as an increased tendency for the animal to gain more complete knowledge of its environment.

## **Histology**

Seven days after ischemia the rats were anesthetized with 3.5% aqueous chloral hydrate (Fluka #00672) and their brains were fixed with 4% paraformaldehyde after transcatheterial perfusion with heparinized saline containing 4% sodium nitrite. Fixed brains were cut into 30  $\mu\text{m}$  sections on a sliding microtome and the sections were stained with cresyl violet. Neuronal cell density was measured by counting viable cells in the total 6 frames (1.0 mm  $\times$  1.0 mm) of left and right CA1 regions of three coronal sections (about 3.3, 3.5, and 3.7 mm caudal to Bregma) for each animal. Viable cells were counted using the Metaview Image Analysis Program (Universal Imaging, West Chester, PA, USA); cell counts were compared to manual determinations by three technicians blinded to the experimental conditions. Neuronal cell density is equivalent to the average number of viable cells in one frame.

## **RNA Extraction**

Mice were killed by cervical dislocation; brain tissue was excised quickly and immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Total RNA was extracted using the TRI REAGENT Kit (Molecular Research Center, Inc., Cincinnati, OH, USA), following the manufacturer's protocol. RNA concentrations were determined by absorption at 260 nm wavelength. Purity was monitored by measuring the ratio of absorbance at 260 nm to that at 280 nm.

## **Microarray mRNA Analysis**

Aliquots of total RNA (10  $\mu\text{g}$  each, as determined from absorbance at 260 nm wavelength and verified by gel ethidium bromide fluorescence intensity) were reverse-transcribed with [ $\alpha$ - $^{32}\text{P}$ ]dCTP incorporated and applied to cDNA microarrays (SuperArray Inc., Bethesda,

MD, USA) containing 23 stress-related genes. The microarrays were autoradiographed for periods varying from 8 h to 7 days at  $-70^{\circ}\text{C}$  on X-ray film (X-OMAT AR, Kodak, and Rochester, NY, USA). A densitometer (Eagle Eye image-processor combined with DNA Scan signal analysis software, Stratagene, San Diego, CA, USA) was used to quantify the signals as area-integrated optical density.

### **Statistical Analyses**

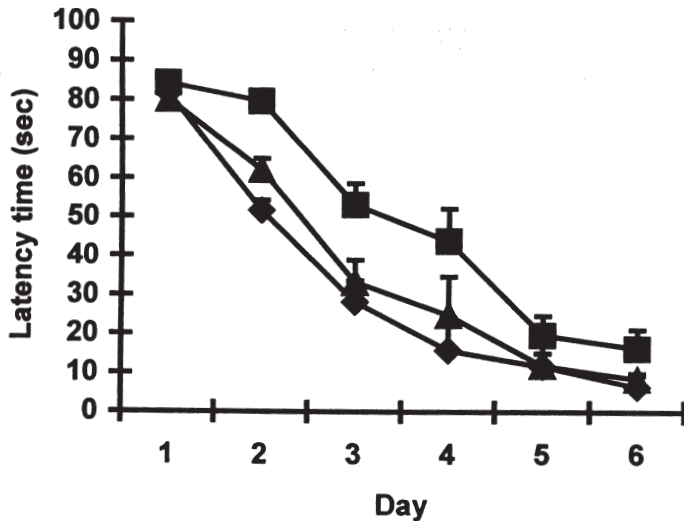
Data for the 4-VO and scopolamine experiments were analyzed by use of Student's *t*-test in which each of the test groups was compared with the control group. Differences between dietary groups were assessed by one-way Analysis of Variance followed by Gabriel's Test. The acceptance level of significance was  $p < .05$  using a two-tailed distribution.

## **RESULTS**

### **Cognitive-Enhancing Activity of YKD Extract following Ischemia/Reperfusion**

Seven days following ischemia induced by 4-VO, rats were trained in the water maze for 5 days and their mean latency times ( $\pm$ SEM) were measured (Figure 1). Latency times for the 4-VO rats were consistently longer than times for either the sham-operated or YKD extract-treated animals, as was the time to reach the platform region during the probe trial on day 6. Ischemic animals receiving YKD extract took slightly longer to swim to the platform than sham-operated animals, but performed just as well in the probe trial. During the probe trial, sham-operated rats spent about 30% of the 90-s trial duration swimming within the quadrant in which the platform had previously been located (data not shown). This finding suggests that these animals remembered the platform position better than ischemic animals, which spent significantly less time in that quadrant (data not shown).

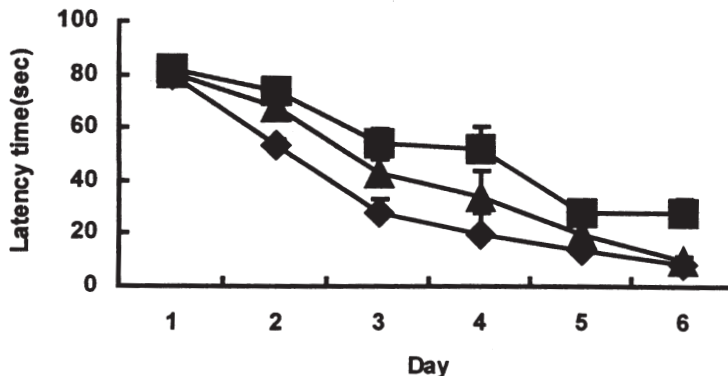
As a model of neurodegenerative memory loss, water maze latency times of mice injected with scopolamine were measured. Sco-



**FIGURE 1.** Comparison of acquisition performance on the Morris water maze task among the three groups of 6-week-old Wistar rats. Beginning one day after the 4-vessel occlusion (4-VO) operation, rats performed four trials per day for 5 days. The probe trial, with platform removed, was conducted on the 6th day. YKD extract (100 mg/kg, i.p.) was injected each day. Data are presented as mean  $\pm$  SEM ( $n = 10$ ).  $\blacklozenge$  = sham;  $\blacktriangle$  = 4-VO+YKD;  $\blacksquare$  = +4-VO.

polamine injection substantially lengthened latency times, whereas animals treated with both scopolamine and YKD extract performed as well as the saline-injected controls (Figure 2). Latency times of the control and scopolamine + YKD groups during the day-6 probe trial were the same, in contrast to the significantly longer latency time required by the scopolamine-injected group. Thus treatment with YKD extract is capable of an essentially complete rescue of scopolamine-induced memory loss as measured by this test. The same set of experiments performed with rats produced substantially the same results (data not shown).

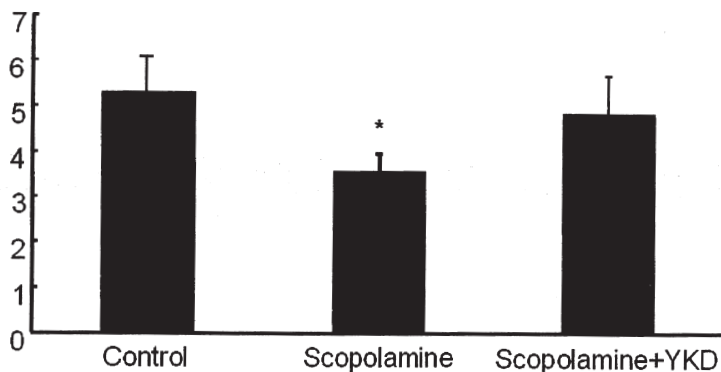
During the fifth acquisition trial, scopolamine-dosed animals spent less time in the target quadrant than the scopolamine/YKD or control groups did (Figure 3). Notably, there was no significant difference between the scopolamine/YKD and control groups, demonstrating that YKD is capable of substantially rescuing rodents from scopolamine-induced memory deficit.



**FIGURE 2.** Comparison of acquisition performance on the Morris water maze task among the three groups of 16-week-old mice. Following scopolamine injection (1 mg/kg, i.p.) on day 1, mice received YKD extract (1000 mg/kg, p.o.) daily and performed four trials per day for 5 days. The probe trial, with platform removed, was conducted on the 6th day. Data are presented as mean  $\pm$  SEM ( $n = 10$ ). ◆ = sham; ▲ = 4-VO+YKD; ■ = +4-VO.

### Neuroprotective Effect of YKD Extract on Global Cerebral Ischemia in vivo

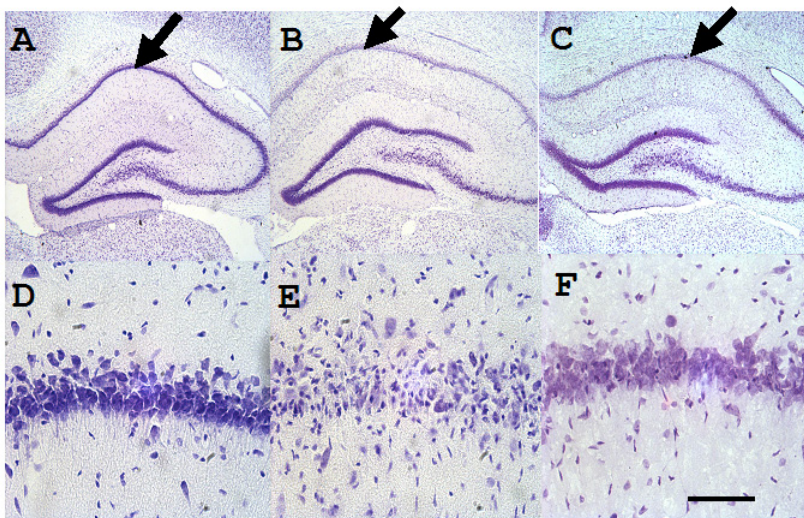
To examine the neuroprotective effect of YKD extract, a dose of 100 mg/kg was injected i.p. into rats 0 and 90 min after the induction of cerebral ischemia. For the ischemia group, 0.89% physiological



**FIGURE 3.** Average number of times during the acquisition trials an animal passed through the quadrant containing the escape platform in the Morris water maze. Thirty min after scopolamine (1 mg/kg) injection, the YKD mice (16-week-old C57BL/6 females) were orally treated with YKD extract (1000 mg/kg). Bars display mean  $\pm$  SEM ( $n = 10$ ). \*Significantly different from the vehicle control group ( $p < .05$ , one-way ANOVA).

saline was injected at a volume of 1.8 ml/kg. When reperfusion is conducted after cerebral ischemia caused by 4-VO, pyramidal neurons in the hippocampus CA1 subfield are the most susceptible to the ischemia and start undergoing cell death 72 h after reperfusion (Pulsinelli & Brierley, 1979). In this study, rats were sacrificed 7 days after reperfusion, the time point by which all signs of neuronal cell damage have become manifest. Dorsal hippocampal tissue sections were stained with cresyl violet to visualize CA1 neurons in the ischemic group, the sham-operated group, and the YKD extract-treated group (Figure 4, A to F).

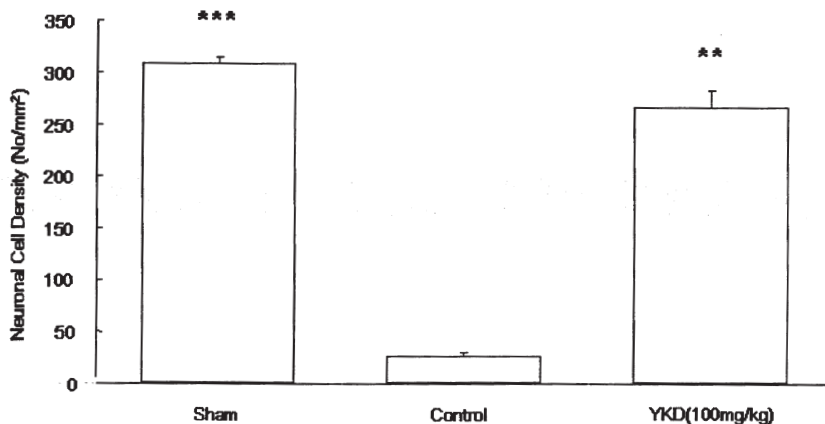
Figure 4A shows the track of CA1 pyramidal neurons in the sham-operated group; most of these neurons have an unchanged (normal) staining pattern (Figure 4B). In the ischemic group, the stratum pyramidal was weakly stained, showing occurrence of neuronal



**FIGURE 4.** Representative photomicrographs of cresyl violet-stained hippocampal regions of either sham-operated 6-week-old male Wistar rats (A,B) or rats that had been subjected to 10 min of ischemia followed by treatment with either saline (C,D) or 100 mg/kg of YKD i.p. (E,F). Boxed regions in A, C, and E are shown in B, D, and F, respectively. The ischemia caused selective and delayed neuronal cell loss in the hippocampal CA1 region (C,D). In contrast, YKD treatment conferred neuroprotection by markedly reducing the number of damaged pyramidal cells in the CA1 subfield (E,F). Scale bar denotes 100  $\mu$ m. (See Color Plate IV at end of issue.)

cell damage within the CA1 subfield (Figure 4C); Figure 4D shows that pyramidal neurons have undergone coagulative cellular changes typical of apoptosis and were damaged with characteristic apparent gliosis. Compared to the ischemic rats, animals administered YKD extract had a significantly reduced number of damaged pyramidal neurons in the CA1 field (Figure 4, E and F). There was no significant difference in body temperature between ischemic and YKD extract treated groups at any time point recorded (data not shown) indicating that neuroprotective effects of YKD extract were not due to a decrease in body temperature.

Normal CA1 pyramidal neurons from three hemispherical sections each having a size of  $1 \times 1$  mm, were counted and averaged (Figure 5). In the ischemic group the viable cell density was  $38 \pm 1.7$  cells/mm<sup>2</sup>, which is far lower than that in the sham group,  $302 \pm 7.4$  cells/mm<sup>2</sup>. In the group injected with YKD extract, viable cells were measured to be  $275 \pm 3.6$  cells/mm<sup>2</sup>. Thus YKD extract rescued 89.3% of the ischemic neurons.



**FIGURE 5.** Neuroprotective effects of YKD (100 mg/kg). Either saline or YKD was orally administered to 6-week-old male Wistar rats following 10 min ischemia. Seven days later, animals were sacrificed and the cell density of CA1 neurons was determined (cells/mm<sup>2</sup>, mean  $\pm$  standard deviation). Statistically significant differences from saline-treated (control) group: \*\* $p < .05$ , \*\*\* $p < .001$ . Sham = sham-treated animals ( $n = 11$ ); control = saline-treated animals following ischemia ( $n = 7$ ); YKD = YKD-treated animals following ischemia ( $n = 6$ ).

### **mRNA Expression Level Changes with Age and Dietary YKD Supplementation**

Microarrays were used to measure mRNA expression levels in mice aged 4, 12, or 24 months and fed either basal or 1% YKD-supplemented diets for 13 weeks. Expression levels of eleven genes were detectable (Table 2); levels of the remaining 12 genes were too low to quantitate.

Expression levels of *Egr1* significantly declined with age (Figure 6), and YKD tended to raise the middle-aged and old animal levels to young animal levels. YKD did not affect *Egr1* levels in young animals.

Expression levels of *Grp78*, which is translated into a heat shock protein, tended to decline with age (Figure 6), and YKD tended to raise the middle-aged and old animal levels to young animal levels.

YKD had a tendency to raise expression levels of *Mkk4* in middle-aged and old animals; the mean level in old animals tended to be less than in younger ones (Figure 6).

Expression levels of *SOD1*, the protein product of which is an important antioxidant enzyme and protector against reactive oxygen species (ROS) damage, were significantly lower in old animals compared to young and middle-aged (Figure 6). YKD significantly raised *SOD1* expression in old animals; although there was also a tendency for it to increase *SOD1* levels in young animals also, it had little effect on middle-aged animal levels.

Expression levels of *Hsf1*, which is translated into a protein factor associated with heat shock proteins, were similar in young and middle-aged animals, but were depressed in old age. YKD tended to lower expression in young and middle-aged animals, but left them unchanged in old animal brains.

Expression levels of *Hsp86*, which is translated into a heat shock protein, tended to decline with age. YKD tended to increase levels of this chaperone in the two older groups.

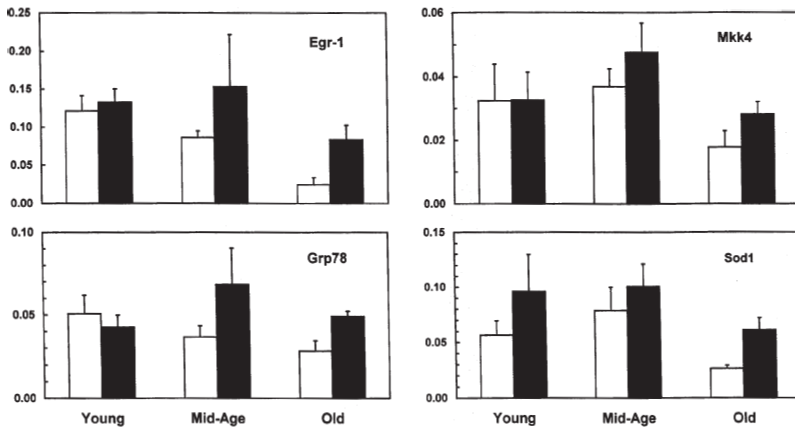
*Trpm2* expression levels are upregulated in tumors; its protein product is involved in apoptosis. There was a tendency for expression levels of *Trpm2* to decrease with age. YKD tended to raise *Trpm2* expression levels in animals of all ages; this tendency approached statistical significance in old animals.

**TABLE 2.** CNS cortical expression levels of genes in female B6D2F1 mice aged 5 (young), 17 (mid-aged), or 28 months (old) fed either basal diet (control) or basal diet supplemented with 1% *youkongo* extract (YKD) for 13 weeks

Gene	mRNA expression relative to $\beta$ -Actin and GAPDH											
	Young control		Young YKD		Mid-Aged control		Mid-Aged YKD		Old control		Old YKD	
	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE
Egr1	0.121	0.020	0.133	0.018	0.087	0.008	0.153	0.069	0.024	0.009	0.084	0.019
Grp78	0.051	0.011	0.043	0.007	0.037	0.007	0.068	0.022	0.028	0.006	0.049	0.003
Hsp86	0.398	0.101	0.292	0.061	0.272	0.068	0.517	0.113	0.218	0.033	0.310	0.050
Mdm2	0.029	0.008	0.028	0.004	0.031	0.003	0.048	0.016	0.017	0.001	0.022	0.001
SOD1	0.056	0.013	0.096	0.034	0.079	0.021	0.100	0.021	0.026	0.003	0.061	0.011
Bax	0.002	0.001	0.007	0.005	0.006	0.003	0.001	0.001	0.000	0.000	0.005	0.005
Hsf1	0.013	0.005	0.007	0.004	0.012	0.005	0.004	0.002	0.002	0.002	0.002	0.002
Mkk4	0.032	0.012	0.033	0.009	0.037	0.006	0.048	0.009	0.018	0.005	0.028	0.004
Trpm2	0.864	0.128	0.787	0.060	0.792	0.084	1.129	0.201	0.694	0.051	1.047	0.052
Cryab	0.167	0.047	0.115	0.021	0.178	0.027	0.161	0.051	0.069	0.013	0.137	0.029

*Notes.* Values are relative to the mean expression of  $\beta$ -Actin and GAPDH.  $n = 5$  for all groups, except for *old controls* ( $n = 3$ ), and for *old YKD* ( $n = 2$ ). Bax = Bel-associated X protein; Cryab =  $\alpha$ B crystallin; Egr1 = early growth response 1; Grp78 = Glucose-regulated protein, 78kD; Hsf1 = heat shock factor 1; Hsp86 = heat shock protein 86; Mdm2 = murine double minute chromosome clone 2; Mkk4 = mitogen-activated protein kinase 4; SOD1 = Cu-Zn superoxide dismutase; Trpm2 = testosterone repressed prostate message 2; GAPDH = glyceraldehyde 3-phosphate dehydrogenase. SE = standard error.





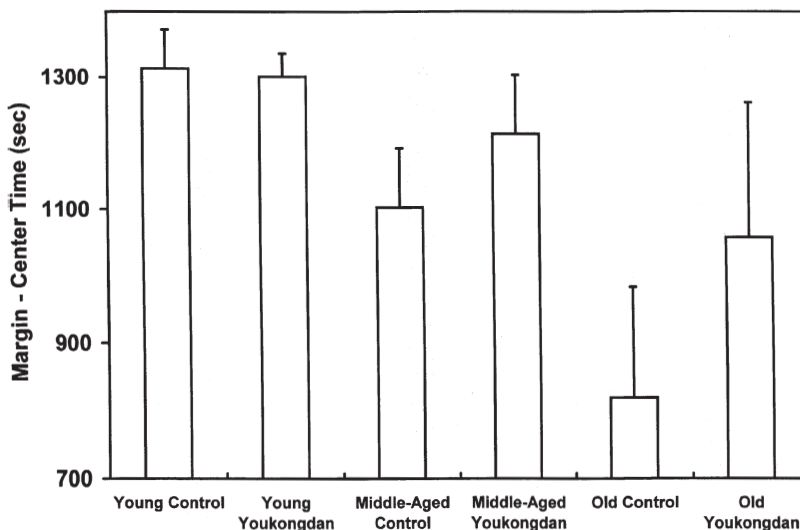
**FIGURE 6.** Levels of Egr1, Grp78, Mkk4, and SOD1 mRNA expression levels in brain cortex of 5 (young,  $n = 5$ ), 17 (mid-age,  $n = 5$ ) and 28 (old  $n = 2$ ) month old mice. Values were densitometrically quantitated and are expressed relative to average of  $\beta$ -actin and GAPDH mRNA expression  $\pm$  standard error. White bars = basal diet; black bars = basal diet plus 1% YKD.

Expression levels of  $\alpha$ B crystallin were similar in young and middle-aged animals, but were depressed in old age. YKD increased the Gadd45 expression in young and old animals, but greatly decreased it in middle-aged animals.

Minimally detectable levels of Bax (Bcl-associated X protein) were measured. YKD apparently increased Bax levels in young and old animals, but may have lowered them in middle-aged animals.

### Open Field Behavior and YKD Dietary Supplementation

Open field locomotor behavior of mice given YKD-supplemented diets was observed. The difference in time a mouse spends at the margin of the enclosure minus the time it spends in the center is a measure of exploratory behavior. This measure decreased with age, but YKD supplementation tended to increase it in old animals (Figure 7). Other measures of locomotor behavior did not show significant changes (data not shown).



**FIGURE 7.** Open field margin minus center time of 5 (young), 17 (mid-age), and 28 (old) month old mice, either controls or *youkongdan*-fed. Values are in seconds  $\pm$  standard error ( $n = 5$  for 1% young and middle-aged animals;  $n = 3$  or 2 for old).

## DISCUSSION

All living organisms suffer from oxidative damage due to ROS, yet the animal brain is often said to be especially sensitive. One reason is its high  $O_2$  consumption; in humans the brain accounts for only a few percent of the body weight, but it consumes about 20% of basal  $O_2$  consumption. Consequently it processes a large amount of  $O_2$  in a relatively small tissue mass. Excessive amounts of ROS are generated during reperfusion following focal ischemia (Kuroda & Siesjo, 1997) and there is extensive evidence that numerous antioxidant compounds are neuroprotective (Behl & Moosmann, 2002).

In the present study, the efficacy of YKD for the prevention of neuronal damage and for the reduction of memory impairment was studied in a Wistar rat model of transient global ischemia and in a murine scopolamine model. Based on the use of YKD in traditional medicine for the treatment of CNS dysfunction, the authors tested potential neuroprotective effects of YKD using the 4-VO model in rats. The results indicate that YKD confers significant neuroprotection

against 10 min of ischemia induced by 4-VO. This is reasonable because several ingredients of YKD have been shown to provide neuroprotection to the brain. Ginseng radix is effective in the prevention and repair of cerebral ischemia (Xuejiang et al., 1999). It has also been shown that ginsenoside, a ginseng component, protects ischemic hippocampal neurons (Um et al., 1997). Tetramethylpyrazine and paeoniflorin, components of *Cnidium* rhizome and peony root, respectively, significantly improved the 8-arm radial maze performance of rats subjected to 2-vessel occlusion (Watanabe, 1997). In the same study, tetramethylpyrazine also significantly improved the radial maze performance of scopolamine-injected rats. Decursin, an acetyl cholinesterase inhibitor occurring in *Angelica gigantis radix*, significantly improved the water maze and passive avoidance test performance of mice injected with scopolamine (Kang et al., 2003). Finally, a decoction of the largest YKD component, *Astragalus membranaceus*, has been shown to alleviate the dyskinesia produced by cerebral ischemia in rats (Quan & Du, 1998).

It has been suggested that neuronal damage in brain ischemia and reperfusion is partly due to oxidative damage caused by free radical formation (Halliwell, 1992). Free radicals are extremely reactive compounds that can react with lipids, enzymes (Frassetto et al., 1997), or DNA to produce various harmful effects (Siesjo et al., 1989). Recent studies have shown that the simultaneous production of NO and superoxide anion within reperfused ischemic cerebral tissue might lead to the formation of cytotoxic peroxynitrite or an oxidant derived from it (Kumura et al., 1996). However, further studies are necessary to determine whether components of YKD other than ginseng have similar neuroprotective effects.

Typically, once cells are induced to undergo apoptosis, they shrink, losing their intrinsic shapes established according to their differentiation. Additionally, as the shrinkage breaks the junctions with surrounding cells, the interaction of the apoptotic cells with adjacent cells is interrupted. As the shrinkage proceeds apoptotic bodies form while the cell membranes seem to blister. As apoptosis advances further, the nuclear chromatin condenses and the nuclear envelope collapses. The morphology of ischemic neurons (Figure 4, C and D) is indicative of apoptosis. Unlike normal neurons from sham-operated animals (Figure 4B), the apoptotic cells from ischemic tissue

are detached from surrounding cells and have lost their characteristic pyramidal morphology, forming a kind of single cell. In contrast, the neuronal cells in the hippocampal CA1 subfields of YKD extract-treated animals have a morphology similar to that of normal cells, as is apparent in Figure 4, E and F.

Scopolamine, a muscarinic antagonist, impairs learning and memory in rodents and humans, especially the processes of learning acquisition and short-term memory (Kopelman & Corn, 1988) by blocking cholinergic neurotransmission. We found that YKD had cognitive-enhancing effects in the scopolamine-induced amnesia model in both rats and mice.

The Morris water maze is a paradigm designed to target hippocampus-dependent spatial learning and is sensitive to deficits accompanying lesions of the hippocampus (Hagan & Beaughard, 1990). Rats subjected to global ischemia show impairment in spatial learning in the water maze that correlates with damage to CA1 hippocampal neurons (Nunn & Jarrad, 1994; Block & Schwarz, 1998). That rats subjected to 4-VO display an increased water maze escape latency in comparison to sham-operated controls and at the same time suffer a substantial loss of CA1 hippocampal neurons indicates that the CA1 neurons of the hippocampus play an important role in spatial learning (Olsen et al., 1994).

Treatment with YKD results in significant protection from the hippocampal neuronal damage induced by 4-VO. In the present experiments the effects of YKD on deficits in spatial learning and memory and the neuronal damage produced by 4-VO suggest that neurons protected from ischemic injury by YKD retain almost normal physiological function. By contrast, ischemic control rats had significantly longer escape latencies in the water maze test compared with sham-operated rats, although the performance of the ischemic rats following YKD treatment was improved in the water-maze test. These results show that the deficit in spatial memory induced by global cerebral ischemia was reduced by YKD. Thus, with a better understanding of the active components of YKD that exert neuroprotective activities, YKD could become an invaluable herbal source for the development of novel neuroprotective drugs.

Dietary YKD induces some potentially beneficial gene expression changes in older mice. Early growth response protein 1 (Egr1)

is a zinc finger transcription factor that stimulates neuronal gene expression. In general, *Egr1* is of critical importance for the beneficial processes of learning, microvascular endothelial cell growth, and neovascularization, as well as the detrimental processes of tumor angiogenesis and growth (Fahmy et al., 2003). The *Egr1* gene is centrally involved in memory consolidation, and is typically up-regulated in several brain regions during the acquisition of new behaviors (Tischmeyer & Grimm, 1999). The age-related decline of *Egr-1* expression measured in the present study (Figure 6) may therefore be related to the decline in the ability of aged animals to learn new behaviors. YKD, by virtue of its ability to raise *Egr-1* expression levels in aged animals, has the potential of improving memory consolidation in the elderly.

*Mkk4* protein levels in human prostate cancer cells are inversely related to the metastatic propensity of the cancer; similarly, normal human ovarian tissue contains higher levels of *Mkk4* than ovarian cancer tissues (Robinson et al., 2003). YKD tended to increase *Mkk4* gene expression levels in both middle-aged and old—but not young—animals and this upregulation may be related to the reported anti-cancer activity of some of its components including astragalus (Sinclair, 1998), crataegus (Min et al., 2000), and ginseng (Shin et al., 2000).

Glucose-regulated protein 78 (*Grp78*) is a chaperone protein involved in assessing the quality of protein synthesis. It has an especially important role during brain ischemia/reperfusion, when it dissociates from PERK, the protein to which it normally binds, and instead sequesters proteins unfolded as a result of the stroke condition (DeGracia et al., 2002). YKD significantly raised the expression level of the *Grp78* gene in the brains of middle-aged and aged mice; presumably the increased levels of *Grp78* resulting from this response to YKD supplementation would be neuroprotective, especially following stroke.

SOD1 is a key cellular antioxidant enzyme that protects the cell from superoxide ion oxidative toxicity by converting superoxide to hydrogen peroxide and molecular oxygen. Damage induced by such oxidative toxicity has been implicated in a number of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), Huntington's disease (Albers & Beal, 2000), and reperfusion damage

following stroke (Maier & Chan, 2002). SOD1 levels were lower in old animals compared to young and middle-aged animals, and YKD restored old-animal SOD1 levels to young-animal levels. The increased levels of SOD1 produced by YKD may potentially slow neurodegeneration in aged animal brains by conferring greater protection against oxidative damage.

The Bax protein is a key signaling protein in the process of apoptosis or programmed cell death. The mitochondrion plays a crucial role in the apoptotic process; without loss of mitochondrial membrane potential, opening of the mitochondrial membrane pores, and release of cytochrome c from the mitochondrion, apoptosis does not occur. The binding of Bax to the outer mitochondrial membrane is the first step in involving the mitochondrion in the apoptotic process. Animal models indicate that decreased levels of apoptosis may be associated with a lessened degree of damage to the brain following stroke (Love, 2003). The only significant change in Bax expression level produced by YKD supplementation was a decrease in middle-aged animals, which suggests it may be lessening neuronal cell loss in animals of this age group.

The protein  $\alpha$ B crystallin is found in many tissues within the CNS, and its levels are increased in some pathological conditions associated with neurodegenerative diseases (van Rijk & Bloemendal, 2000). YKD supplementation significantly increased the mRNA expression for this protein, but the authors did not determine how this increase affected levels of the protein. In any case, the tendency of YKD treatment in older animals to increase exploratory behavior argues against a relation between increased expression of  $\alpha$ B crystallin gene expression and neurodegeneration in this model.

Although YKD extract treatment produced substantial and significant improvement in animals subjected to ischemia and in memory-impaired animals, chronic YKD supplementation in the diets of old animals at the one dose tested resulted in less notable improvement. These results indicate that YKD extract may prove to be beneficial in the treatment of stroke and memory impairment. YKD dietary supplementation seems to produce some generally beneficial changes in the CNS expression levels of several stress-related genes; however, although the tendency toward improvement in exploratory behavior is suggestive, additional work is needed to demonstrate more

definitively that these changes translate into preventive effects on neurodegeneration.

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