Neuroprotective Effects of Guh-Poong-Chung-Sim-Hwan on Focal Cerebral Ischemia in Rats

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This study was designed to investigate the neuroprotective effects of Guh-Poong-Chung-Sim-Hwan(GCH) on ischemia induced by middle cerebral artery occlusion(MCAO) in Sprague-Dawley rats. The effects of GCH administration on the size of the brain infarct and the functional status of the rats after ischemia were examined, as well as the expression of COX-2 in acute phase. The recovery of motor functions for 7 days and the brain infarct were examined to find out the delayed effects of daily GCH-administration as well. In conclusion, we found that GCH reduced both functional deficits and brain damage in the MCAO rat model of stroke. In addition, high doses of GCH reduced COX-2 expression in the penumbra. It is well known that herbal medication including GCH is very safe for humans. Accordingly, our results support the clinical use of this GKM for the treatment of stroke and offer the possibility that a potent neuroprotective agent could be developed from Korean herbal medicines.

Key words : Guh-Poong-Chung-Sim-Hwan(GCH), middle cerebral artery occlusion(MCAO), brain infarct, COX

Introduction

Brain ischemia is the third leading cause of death after heart disease and cancer[3]. It is characterized by acute fainting, unconsciousness, excessive phlegm, hemiparalysis, dysphasia, facial palsy, and motor disorders. Clinical experiences and therapeutic theory have been integrated into Genuine Korean Medicine (GKM) over thousands of years[7,8]. Based on this accumulated knowledge, acupuncture and many herbal decoctions have been used for the treatment of stroke.

Guh-Poong-Chung-Sim-Hwan (GCH) is a novel decoction developed on the basis of our preliminary data and GKM theory regarding the treatment of brain ischemia[7,8]. It is composed of fifteen different herbal medicines: Ginseng Radix, Angelicae Gigantis Radix, Paeoniae Radix, Cnidii Rhizoma, Curcumae Radix, Gastrodiae Rhizoma, Saposhnikovia Radix, Ostericii Radix, Angelicae Tenuissimae Radix, Menthae Herba, Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, Gardeniae Fructus, and Rhei Radix. In the terminology of GKM, Ginseng Radix is used to tonify the Qi. Angelicae Gigantis Radix, and Paeoniae Radix are used to tonify the blood. Cnidii Rhizoma, and Curcumae Radix are used to invigorate the blood flow. Gastrodiae Rhizoma is used to extinguish wind and stop tremors. Saposhnikovia Radix, Ostericii Radix, Angelicae Tenuissimae Radix, and Menthae Herba are used to release external conditions and expel the outer wind. Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, and Gardeniae Fructus are used to clear heat and dry dampness. Rhei Radix is used to purge inner heat[18].

Cyclooxygenase (COX), also known as prostaglandin H synthase, is the rate-limiting enzyme in the metabolism of arachidonic acid to prostanoids (prostaglandins and thromboxanes). There are two different COX genes: COX-1, which is constitutively expressed under physiological conditions, and COX-2, which is inducible in response to mitogens, endotoxins, and cytokines[25]. COX-2 is rapidly induced in inflamed tissues, and its reaction products are responsible for many of the cytotoxic effects of inflammation[21]. It has been reported that brain ischemia induces high expression levels of COX-2 in neurons and plays an important role in ischemic neuronal cell death, significantly contributing to enlargement of the infarction[14].

This study was designed to investigate the neuroprotective effects of GCH on ischemia induced by middle cerebral artery occlusion (MCAO) in Sprague-Dawley rats. The effects of GCH administration on the size of the brain infarct and the functional status of the rats after ischemia were examined, as well as the expression of COX-2 in acute phase. The recovery of motor functions for 7 days and the brain

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infarct were examined to find out the delayed effects of daily GCH-administration as well.

Materials and Methods

1. Effects of Guh-Poong-Chung-Sim-Hwan in acute phase

1) Sample preparation and administration

The components of Guh-Poong-Chung-Sim-Hwan (GCH) are fifteen herbal medicines (Table 1). It was extracted with distilled water at 100℃. The yield of the dried and pulverized extract was 28.2%. The sample was dissolved in distilled water prior to use. The GCH extract was administered to the rats (n=9) the treated group (50 or 200 mg/kg, p.o.) at 0 and 2 h after occlusion of the middle cerebral artery. Distilled water (3 ml/kg, p.o.) was administered to the rats (n=9) the control group.

Table 1. Components of Guh-Poong-Chung-Sim-Hwan

Drug name (藥名)	Scientific Name	% (w/w)
Coptidis Rhizoma (黃連)	*Coptis japonica*	6.45
Phellodendri Cortex (黃柏)	*Phellodendron amurense*	3.23
Scutellariae Radix (黃芩)	*Scutellaria baicalensis*	9.68
Gardeniae Fructus (梔子)	*Gardenia jasminoides*	3.23
Cnidii Rhizoma (川芎)	*Cnidium officinale*	9.68
Angelicae Gigantis Radix (當歸)	*Angelica gigas*	9.68
Paeoniae Radix (白芍藥)	*Paeonia lactiflora*	6.45
Curcumae Rhizoma (鬱金)	*Curcuma longa*	6.45
Saposhnikovia Radix (防風)	*Saposhnikovia divaricata*	9.68
Gastrodiae Rhizoma (天麻)	*Gastrodia elata*	6.45
Ginseng Radix (人參)	*Panax ginseng*	6.45
Ostericii Radix (羌活)	*Ostericum koreanum*	6.45
Menthae Herba (薄荷)	*Mentha arvensis*	3.23
Rhei Rhizoma (大黃)	*Rheum palmatum*	6.45
Angelicae Tenuissimae Radix (藁本)	*Angelica tenuissima*	6.45
Total		100

2) Experimental procedure

All surgical procedures were conducted according to the animal welfare guidelines of the National Institutes of Health and the Korean Academy of Medical Sciences. Male Sprague-Dawley rats (290-310 g) were housed under controlled temperature conditions (22 ± 2℃), with constant humidity and a 12-h light/dark cycle (lights on, 07:00-19:00). Food and water were available *ad libitum*. Prior to surgery, the rats were fasted overnight, but had free access to water. The rats were pre-anesthetized with 5% isoflurane in N_2O and O_2 and were maintained with 1-2% isoflurane. The rectal temperature was maintained at 37.0 ± 0.5℃ throughout the experiment with a heating lamp and blanket system (Harvard Apparatus, Holliston, MA, USA). Permanent focal ischemia was produced by an intraluminal suture method described by Zea Longa[26]. The left carotid bifurcation was exposed through a midline neck incision. The external carotid artery (ECA) was ligated and then cut just proximal to the external carotid bifurcation. The common carotid artery (CCA) and internal carotid artery (ICA) were temporarily occluded with microvascular clips. A 4-0 nylon monofilament (0.30-mm diameter) with a rounded tip and a distal cylinder of silicone rubber was introduced into the external carotid artery. The correct placement of the suture was established when the suture was inserted at least 18-19 mm from the CCA/ICA bifurcation. The body temperature was maintained at 37.0 ± 0.5℃ from 0 to 6.5 h after the occlusion.

3) Neurological examination

Neurological deficits were evaluated by a blinded observer at 24 h after ischemia. A clinical score scale of 0 to 3 was used to assess the neurological performance, as described below[2,24]. The three major tests described below were conducted sequentially. The functional level of each rat was scored according to its ability to perform the elicited behavior at each subsequent step of the testing sequence.

(1) Forelimb flexion

When held by the tail and suspended about 0.5 m above a rubber mat, a normal rat will extend both forelimbs toward the mat. Rats that performed this behavior and had no other neurological deficits were assigned a clinical score of 0. Rats with cerebral infarction consistently flexed the forelimb contralateral to the injured hemisphere; the posture varied from mild wrist flexion and shoulder adduction, with extension at the elbow, to severe posturing, with full flexion at the wrist, elbow, and adduction with internal rotation of the shoulder. Rats that showed these features were assigned a score of 1. Rats that exhibited flexion with extreme body twisting and curling were assigned a clinical score of 1.5.

(2) Resistance to lateral push

Rats were placed on a grapple rubber mat surface with the tail held in the tester's hand; a gentle lateral pressure was applied behind the rat's shoulder until the forelimbs slid several inches. This maneuver was repeated several times in each direction. Normal rats showed equal resistance to pressure in both directions. Rats with severe neurological dysfunction had consistently reduced resistance to lateral pressure toward the paretic side and were assigned a score of 2. Rats that were less resistant to lateral pressure and showed no circling, but did

stagger toward the paretic side, were assigned a score of 2.5.

(3) Circling

Rats were allowed to roam freely and were observed for circling behavior. Rats that consistently circled toward the paretic side were assigned a clinical score of 3.

4) Motor behavioral evaluation

After the neurological examination, the animals were tested with two different motor tasks : the forelimb foot fault placing test, which is used to test forelimb function, and the parallel bar crossing test, which is used to test hindlimb function. The forelimb foot fault placing test was performed as previously described[1,5,10,15,20,23,25]. In brief, the foot fault placing apparatus consisted of an elevated (1 m) wire grid surface (10 × 110 cm, with 9cm 2 openings between the wires and 1.0 mm-diameter grid wires) connected to platforms at each end (15 × 20 cm)[4]. In each trial, the animal was placed on the grid and forced, by prods and noise, to cross the grid surface for 1 min. The inaccurate placement of the fore- or hindlimb allows the limb to fall through one of the openings in the grid. These mistakes were considered "foot faults." The number of contralateral forelimb foot faults made per meter in 1 min was calculated and scored for each animal(Table 2). The parallel bar test is a particularly sensitive means of detecting impairment of hindlimb coordination[5,13]. The parallel bar apparatus consisted of two parallel wooden rods (1.0 cm diameter, 115 cm long), with an inter-rod distance of 2.5 cm, connected to platforms at each end (15 × 50 cm). The number of times that the animal placed two hind paws on one rod, dropped a hind paw below the rod, or fell or swung under the rods was recorded. The number of errors made per meter in 1 min were calculated and scored. The motor behavior performance was scored from 0 (excellent) to 5, depending on the time required, the errors made, and the amount of prodding or help needed during the performance of the tests (Table 2).

Table 2. Scoring system for motor behavior

Score	Criteria	
	Parallel bar	Foot fault
0	No error	No fault
1	Less than 1 error made within 1 min per meter length	Less than 1 fault made within 1 min per meter length
2	Equal or more than 1 error made within 1 min per meter length	Equal or more than 1 fault
3	More than 2 errors made within 1 min per meter length	More than 2 faults
4	More than 3.5 errors made within 1 min per meter length	More than 3 faults
5	More than 6 errors or cannot traverse	Cannot move on

5) Tissue preparation and measurement of infarct volume

The rats were sacrificed with an overdose of chloral hydrate at 24 h after reperfusion. The brains were removed quickly and cut into six 2 mm-thick coronal sections. The sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC Sigma, USA) in saline for 30 min at 37℃ and fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 24 h. The TTC-stained sections were analyzed using a computerized image analysis system (Optimas Media Cybernetics, USA) for measurement of the infarct volume. The infarct area (mm^2) of each brain slice was determined, and then the infarct volume (mm^3) was calculated from the sum of the infarct areas of the six slices multiplied by the thickness (2 mm). The brain slices were then sectioned at a thickness of 40 ㎛ with a sliding microtome (HM440, Zeiss, Germany), and the sections were used for immunohistochemistry.

6) Examination of COX-2 expression

For detection of COX-2 expression in brain, antigen-specific immunohistochemistry was performed. In brief, 40 ㎛ sections were incubated in 10% hydrogen peroxide for 10 min and rinsed twice with 0.05 M PBS. The sections were incubated in 1% Triton X-100 in PBS for 15 min and rinsed twice with 0.05 M PBS. After incubation with 0.5% bovine serum albumin in 0.1 M PBS for 30 min, the sections were incubated overnight with a goat anti-rat COX-2 polyclonal antibody (1:500 Santa Cruz Biotechnology, USA) at room temperature. Then the sections were rinsed three times with 0.05 M PBS, incubated with biotinylated anti-goat antibody (1:100) for 1 h, rinsed again, and then incubated with avidin-biotin-horseradish peroxidase complex (1:100) for 1 h. The peroxidase reaction was developed with 0.02% 3,3'-diaminobenzidine in 50 mM Tris-HCl (pH 7.6) for 5 min. Finally, the sections were washed in water and mounted onto gelatinized glass slides.

7) Statistical Analysis

The results were expressed as means± standard error of the mean (SEM). One-way ANOVA with Tukey's multiple comparison test was used to compare the differences between the groups. Values of $p < 0.05$ were considered significant.

2. Effects of Guh-Poong-Chung-Sim-Hwan in functional recovery for 7 days

1) Sample administration

The extract of Guh-Poong-Chung-Sim-Hwan (GCH) was dissolved in distilled water prior to use. The GCH extract was administered to the rats (n=7, 200 mg/kg, p.o.) at 0 and 2 h after occlusion of the middle cerebral artery and once per a

day for consecutive 7 days. Distilled water (3 ml/kg, p.o.) was administered to the rats in the sham-operated group (n=7) and control group (n=7) at 0 and 2 h after operation and once per a day for consecutive 7 days.

2) Experimental procedure

All surgical procedures were conducted as previously described. In brief, rats were pre-anesthetized with 5% isoflurane in N_2O and O_2 and were maintained with 1-2% isoflurane. The rectal temperature was maintained at 37.0 ± 0.5℃ throughout the experiment with a heating lamp and blanket system (Harvard Apparatus, Holliston, MA, USA). Permanent focal ischemia was produced by an intraluminal suture method described by Zea Longa[26]. The body temperature was maintained at 37.0 ± 0.5℃ from 0 to 6.5 h after the occlusion.

3) Neurological examination

Neurological deficits were evaluated daily after 24 h of ischemia for 7 consecutive days by a blinded observer as previously described. A clinical score scale of 0 to 3 was used to assess the neurological performance, as described below[2,24]. The three major tests described below were conducted sequentially. The functional level of each rat was scored according to its ability to perform the elicited behavior at each subsequent step of the testing sequence.

4) Motor behavioral evaluation

After the neurological examination, the animals were tested with twodifferent motor tasks: the forelimb foot fault placing test, which is used to test forelimb function, and the parallel bar crossing test, which is used to test hindlimb function. Both examinations were evaluated daily for 7 consecutive days by a blinded observer as previously described.

5) Tissue preparation and measurement of infarct volume

The rats were sacrificed with an overdose of chloral hydrate at 7 days after motor behavioral evaluation. The brains were removed quickly and cut into six 2mm-thick coronal sections. The sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC Sigma, USA) in saline for 30 min at 37℃ and fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 24 h. The TTC-stained sections were analyzed using a computerized image analysis system (Optimas Media Cybernetics, USA) for measurement of the infarct volume. The infarct area (mm^2) of each brain slice was determined, and then the infarct volume (mm^3) was calculated from the sum of the infarct areas of the six slices multiplied by the thickness (2 mm).

6) Statistical Analysis

The results were expressed as means± standard error of the mean (SEM). One-way ANOVA with Tukey's multiple comparison test was used to compare the differences between the groups. Values of $p < 0.05$ were considered significant.

Results

1. Effects of Guh-Poong-Chung-Sim-Hwan in acute phase

1) Neurological examination

The rats in the vehicle-treated, control group showed obvious neurological deficits including hemiplegia and postural abnormalities, such as forelimb flexion, extreme body twisting, diminished resistance to lateral push, and/or circling behavior. The clinical score of control group was 2.9 ± 0.1 (Fig. 1). In contrast, hemiplegia and postural abnormalities were milder in the GCH-treated rats. The clinical scores were 2.8 ± 0.1 and 2.2 ± 0.1 for the groups treated with 50 mg/kg and 200 mg/kg GCH, respectively (Fig. 1). The clinical score measured in the group treated with 200 mg/kg GCH was significantly lower than that of the control group ($p < 0.05$).

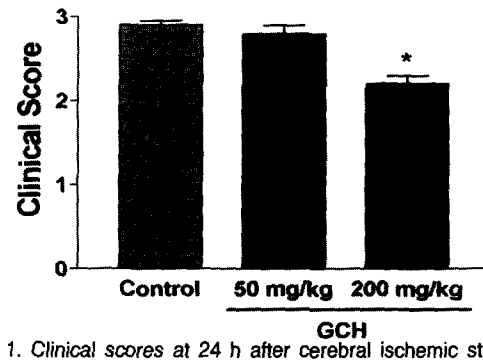

Fig. 1. Clinical scores at 24 h after cerebral ischemic stroke in rats treated with vehicle alone (control group) or with Guh-Poong-Chung-Sim-Hwan (GCH, 50 mg/kg or 200 mg/kg). Values are means ± SEM (*p < 0.05 compared with the control and GCH 50 mg/kg groups).

2) Motor functional performance

Forelimb function was determined using the forelimb foot fault placing test. The animals in the vehicle-treated control group showed a high level of impairment of motor function, with a mean foot fault score of 4.8 ± 0.2. Although the rats that received 50 mg/kg of GCH did not show an improvement compared with controls (4.8 ± 0.1), the rats treated with 200 mg/kg of GCH had significantly reduced ischemia-induced impairment of forelimb function (3.8 ± 0.1, $p < 0.05$; Fig. 2A). The parallel bar test was used to examine the hindlimb motor function. The mean score of the vehicle-treated, control group was 4.7 ± 0.2. The administration of 50 mg/kg and 200 mg/kg GCH improved the parallel bar test scores to 4.5 ± 0.2 and 3.6 ± 0.3, respectively. The group treated with 200 mg/kg GCH showed a statistically significant reduction of ischemia-induced

hindlimb motor impairment compared with the control group and the group treated with 50 mg/kg GCH (Fig. 2B).

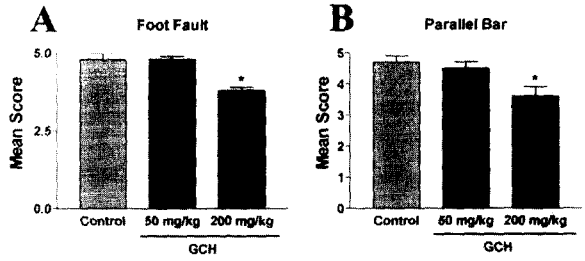

Fig. 2. Motor performance scores at 24 h after cerebral ischemic stroke in rats treated with vehicle alone (control group) or with Guh-Poong-Chung-Sim-Hwan (GCH, 50 mg/kg and 200 mg/kg). In both the forelimb foot placement test (A) and the parallel bar test (B), impairment of motor function was significantly reduced in the GCH-treated ischemic rats (200 mg/kg) compared with the control group ($p < 0.05$). Values are means ± SEM (*$p < 0.05$ compared with the control and GCH 50 mg/kg groups).

3) Infarct volume

The extent of infarction caused by MCAO was quantified in control and GCH-treated rats. Coronal sections (2 mm) were obtained by slicing the brain at 4, 6, 8, 10, 12, and 14 mm from the rostral extremity of the frontal cortex and staining with TTC. Normal tissue stained red with TTC, and the infarcted regions remained white. The coronal sections revealed regions that were effectively protected by treatment with GCH (Fig. 3, arrows). The infarcted area extended from the core regions (caudoputamen, parietal cortex, and temporal cortex) to the peripheral regions (penumbra). In the group treated with 200 mg/kg GCH, the infarct volume was significantly smaller (203.1 ± 40.2 mm^3 $p < 0.05$) compared with that of the control group (377.8 ± 32.6 mm^3). The group that received 50 mg/kg GCH also had a smaller infarct volume than did the control group (352.8 ± 43.9 mm^3), but the difference did not reach significance (Fig. 4).

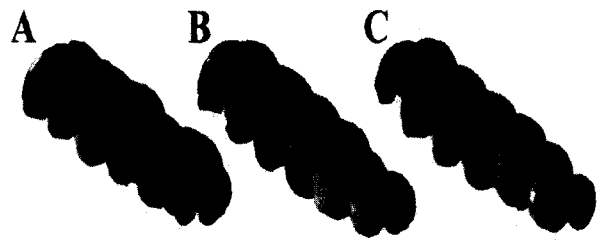

Fig. 3. Representative TTC-stained coronal brain slices of control (A), 50 mg/kg Guh-Poong-Chung-Sim-Hwan (GCH)-treated (B), and 200 mg/kg GCH-treated (C) rats at 24 hour after MCA occlusion. The coronal sections were obtained 4, 6, 8, 10, 12, and 14 mm from the rostral extremity of the frontal cortex.

4) COX-2 expression

MCAO resulted in highly elevated expression levels of COX-2 in thepenumbra of the focal ischemic lesions compared with expression in normal brain tissue (neocortex, Fig. 5), which was consistent with a previous report showing that elevated expression of COX-2 in the penumbra significantly contributed to the enlargement of focal infarctions[14]. In a normal animal (Fig. 5A and D), COX-2 expression was observed on both sides of the neocortex. MCAO induced elevated COX-2 expression in the ipsilateral neocortex (Fig. 5B, arrow) compared with that in the contralateral neocortex. In GCH-treated rats (200 mg/kg), ischemia-induced COX-2 expression was effectively reduced in the ipsilateral neocortex (Fig. 5C and G) compared with that in vehicle-treated rats (Fig. 5B and E).

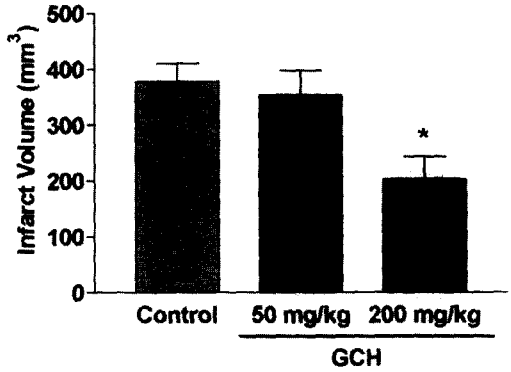

Fig. 4. Infarct volume at 24 h after cerebral ischemic stroke treated with vehicle alone (control group) or Guh-Poong-Chung-Sim-Hwan (GCH, 50 mg/kg or200 mg/kg). Values are means ± SEM (*$p < 0.05$ compared with the control and GCH 50 mg/kg groups).

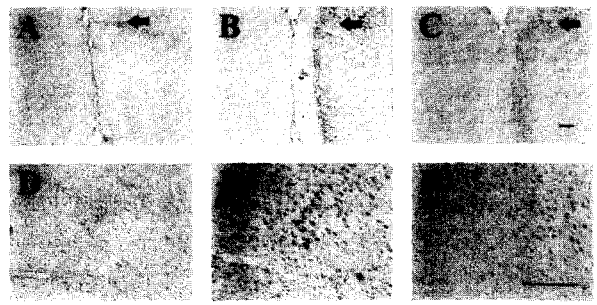

Fig. 5. COX-2 immunostaining in the neocortex at 24 h after MCA occlusion. Representative sections were obtained from a normal rat (A and D), a rat treated with vehicle alone (control, B and E), and a rat treated with Guh-Poong-Chung-Sim-Hwan(GCH, 200 mg/kg; C and F). The regions indicated by arrows in A, B, and C are magnified in the lower images (D, E, and F, respectively). The magnifications of A, B, and C are 100X and those of D, E, and F are 400X. Bar = 100 ㎛.

2. Effects of Guh-Poong-Chung-Sim-Hwan in functional recovery

1) Neurological examination

Neurological examination was measured for 7 days. The rats in the vehicle-treated, sham-operated group did not show any neurological deficit for the whole 7 days. However, control group showed obvious neurological deficits including

hemiplegia and postural abnormalities, such as forelimb flexion, extreme body twisting, diminished resistance to lateral push, and/or circling behavior. The clinical scores of control group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 2.9 ± 0.1, 2.1 ± 0.1, 2.0 ± 0.0, 2.0 ± 0.0, 2.1 ± 0.1, 2.0 ± 0.0, and 2.0 ± 0.0, respectively (Fig. 6). In contrast, hemiplegia and postural abnormalities were milder in the GCH-treated rats. The clinical scores of GCH group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 1.9 ± 0.4, 1.7 ± 0.3, 2.0 ± 0.0, 2.0 ± 0.0, 2.0 ± 0.0, 1.4 ± 0.4, and 1.4 ± 0.4, respectively (Fig. 6). The clinical scores measured in the group treated with 200 mg/kg GCH at 1st day and the sham-operated group at all days were significantly lower than that of the control group ($p < 0.05$).

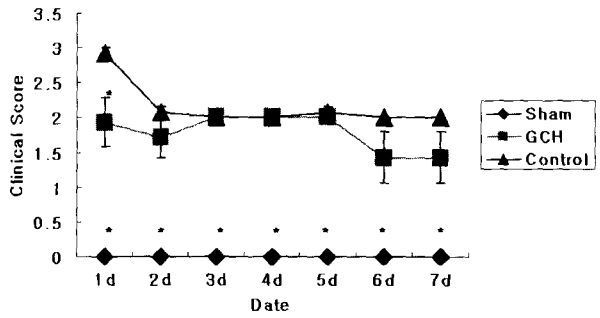

Fig. 6. Clinical scores in rats treated with vehicle alone (sham-operated group (Sham) and stroke-induced control group (Control)) and with Guh-Poong-Chung-Sim-Hwan (GCH, 200 mg/kg) for 7 days. Values are means ± SEM (*p < 0.05 compared with the control group).

2) Motor functional performance

Forelimb function was determined using the forelimb foot fault placing test. The foot fault scores of sham group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 3.0 ± 0.3, 2.8 ± 0.2, 2.8 ± 0.2, 3.0 ± 0.0, 3.0 ± 0.0, 3.0 ± 0.0, and 3.0 ± 0.0, respectively. The foot fault scores of control group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 4.7 ± 0.3, 4.3 ± 0.4, 3.5 ± 0.2, 3.5 ± 0.2, 3.5 ± 0.2, 3.0 ± 0.0, and 3.0 ± 0.0, respectively. The foot fault scores of GCH-treated group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 3.3 ± 0.2, 3.6 ± 0.2, 3.3 ± 0.2, 3.7 ± 0.2, 3.6 ± 0.2, 3.0 ± 0.0, and 3.0 ± 0.0, respectively. The foot fault scores measured in the group treated with 200 mg/kg GCH at 1st day and the sham-operated group at 2nd day were significantly lower than that of the control group ($p < 0.05$; Fig. 7).

The parallel bar test was used to examine the hindlimb motor function. The parallel bar scores of sham group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 2.8 ± 0.2, 2.8 ± 0.2, 2.8 ± 0.2, 3.0 ± 0.0, 3.0 ± 0.0, 3.0 ± 0.0, and 3.0 ± 0.0, respectively. The parallel bar scores of control group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 4.5 ± 0.3, 4.2 ± 0.4, 3.7 ± 0.2, 3.5 ± 0.2, 3.5 ± 0.2, 3.0 ± 0.0, and 3.0 ± 0.0, respectively. The parallel bar scores of GCH-treated group for 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day were 3.7 ± 0.2, 3.4 ± 0.2, 3.3 ± 0.2, 3.7 ± 0.2, 3.4 ± 0.2, 3.0 ± 0.0, and 3.0 ± 0.0, respectively. The parallel bar scores measured in the sham-operated group at 1st, 2nd and 3rd days were significantly lower than that of the control group ($p < 0.05$; Fig. 8).

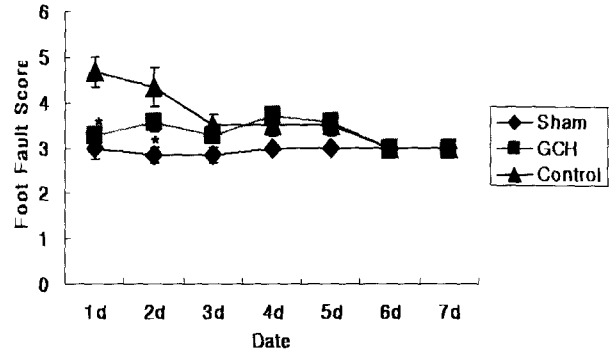

Fig. 7. Motor performance scores for the forelimb foot placement test in rats treated with vehicle alone (sham-operated group (Sham) and stroke-induced control group (Control)) and with Guh-Poong-Chung-Sim-Hwan (GCH, 200 mg/kg) for 7 days. Values are means ± SEM (*p < 0.05 compared with the control group).

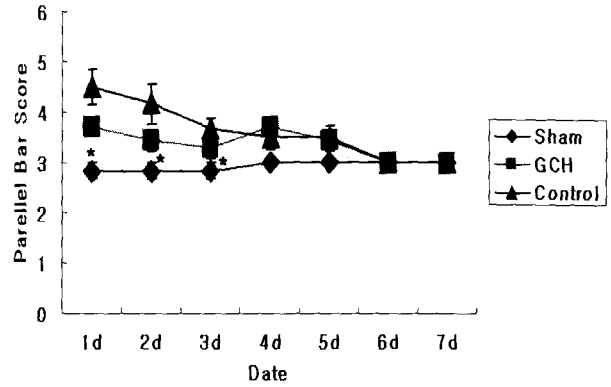

Fig. 8. Motor performance scores for the parallel bar test in rats treated with vehicle alone (sham-operated group (Sham) and stroke-induced control group (Control)) and with Guh-Poong-Chung-Sim-Hwan(GCH, 200 mg/kg) for 7 days. Values are means ± SEM.

3) Infarct volume

The extent of infarction caused by MCAO was quantified in control and GCH-treated rats. Coronal sections (2 mm) were obtained by slicing the brain at 4, 6, 8, 10, 12, and 14 mm from the rostral extremity of the frontal cortex and staining with TTC. Normal tissue stained red with TTC, and the infarcted regions remained white. The coronal sections revealed regions that were effectively protected by treatment with GCH. The infarcted area extended from the core regions(caudoputamen, parietal cortex, and temporal cortex) to the peripheral regions

(penumbra).

In the group treated with GCH (200 mg/kg), the infarct volume was significantly smaller (188.5 ± 32.7 mm^3 $p< 0.05$) compared with that of the control group (386.8 ±30.1 mm^3) (Fig. 9).

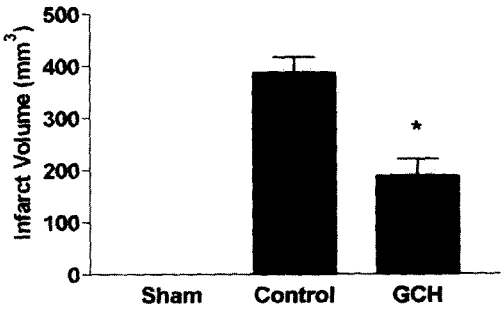

Fig. 9. Infarct volume at 7 days after cerebral ischemic stroke treated with vehicle alone (control group) or Guh-Poong-Chung-Sim-Hwan (GCH, 200 mg/kg). Values are means ± SEM (*p < 0.05 compared with the control group).

Discussion

In the present study, we demonstrate for the first time that the *in vivo* administration of GCH leads to functional improvement after ischemia. The administration of high-dose GCH (200 mg/kg) significantly reduced the clinical score of the treated rats compared with the vehicle-treated, control group ($p < 0.05$), reflecting less severe neurological deficits after ischemia.

We used two motor tasks to provide a summary of motor performance following focal ischemia in rats: forelimb foot placement and parallel bar crossing. The forelimb foot placement test was used to examine the ability of sensorimotor programming of the forelimb to respond to visual, tactile, and proprioceptive stimuli. This test has been studied in other models of stroke[10,15,20,22,23]. The parallel bar crossing test, which depends on the acquisition of motor skills, was used as a sensitive detector of impairment of hindlimb coordination[16]. Rats that received the high dose of GCH performed better on both tests.

The brain infarction in the control group extended from a core region (in the caudoputamen, parietal cortex, and temporal cortex) into a penumbral region, where it is thought that appropriate treatment could prevent permanent neuronal loss. In this study, the GCH-treated group had a significantly reduced mean infarct volume ($p < 0.05$) compared with that of the control group. In summary, these results demonstrate that the administration of the Genuine Korean medicine GCH reduced the ischemia-induced neurological and motor deficits after MCAO in rats and also reduced the extent of brain tissue damage.

Under normal conditions, a subpopulation of neurons expresses COX-2; the other brain cells, including microglia, astrocytes, and vascular cells (both endothelium and smooth muscle cell) do not normally express significant levels of COX-2. COX-2 expression is upregulated by inflammatory stimuli such as ischemia and hypoxia[19]. It has been reported that after focal cerebral ischemia produced by MCAO, COX-2 immunoreactivity was observed in infiltrating neutrophils, vascular cells, and neurons located at the border of the infarct[9]. The administration of a selective COX-2 inhibitor at 6 h after induction of ischemia was found to decrease the volume of the infarction[17,19]. In a model of stroke using permanent and transient MCAO, COX-2 expression was not induced in the ischemic core (lateral striatum), but only in the penumbral area of the MCA cortex[11]. The upregulation of COX-2 began at 6 h after ischemia, reached a maximum at 1224 h, and subsided at 48 h. Our results showed that COX-2 expression was elevated at 24 h in the MCA cortex (neocortex) in the vehicle-treated, control group. In the high-dose GCH-treated group, COX-2 expression was inhibited in the neocortex, compared with that in the vehicle-treated controls. Although the factors responsible for the cytotoxicity of COX-2 have not been clearly defined, the following mechanisms have been implicated. Cerebral ischemia elicits the release of arachidonic acid and increases the levels of cyclooxygenase products such as prostaglandins, thromboxanes, and free radicals[11]. Those products are believed to be involved in the pathogenesis of cerebral ischemic injury. The COX-2 reaction products also contribute to N-methyl-D-asparate-induced neuronal injury and the pathogenic effects of nitric oxide after ischemia[6,12].

In conclusion, we found that GCH reduced both functional deficits and brain damage in the MCAO rat model of stroke. In addition, high doses of GCH reduced COX-2 expression in the penumbra. It is well known that herbal medication including GCH is very safe for humans. Accordingly, our results support the clinical use of this GKM for the treatment of stroke and offer the possibility that a potent neuroprotective agent could be developed from Korean herbal medicines.

References

1. Barth TM, Jones TA, Schallert T. Functional subdivisions of the rat somatic sensorimotor cortex. Behav Brain Res 39: 7395, 1990.
2. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion:

evaluation of the model and development of a neurologic examination. Stroke 17: 472476, 1986.

3. Bonita R. Epidemiology of stroke. Lancet 339: 342-344, 1992.
4. Ding Y, Zhou Y, Lai Q, Li J, Gordon V, Diaz FG. Long-term neuroprotective effect of inhibiting poly(ADP-ribose) polymerase in rats with middle cerebral artery occlusion using a behavioral assessment. Brain Res a; 915(2): 210-217, 2001.
5. Ding Y, Yao B, Lai Q, McAllister JP. Impaired motor learning and deffuse axonal damage in motor and visual systems of the rat following traumatic brain injury. Neurol Res b; 23: 193202, 2001.
6. Hewett SJ, Uliasz TF, Vidwans AS, Hewett JA. Cyclooxygenase-2 contributes to N-methyl--aspartate-mediated neuronal cell death in primary cortical cell culture. J Pharmacol Exp Ther 293: 417425, 2000.
7. Hur J. Dong-Uei-Bo-Gam (東醫寶鑑), 1st edn, Seoul: Dae-Seong, pp. 56-92, 1992.
8. Hwang DY. Jeung-Maek-Bang-Yak-Hap-Pyun (證脈方藥合編), 5th edn, Seoul: Nam-San-Dang, pp. 52-53, 1992.
9. Iadecola C, Forster C, Nogawa S, Clark HB, Ross ME. Cyclooxygenase-2 immunoreactivity in the human brain following cerebral ischemia. Acta Neuropathol 98: 914, 1999.
10. Jones TA, Chu CJ, Grande LA, Gregory AD. Motor skills training enhances lesion-induced structural plasticity in the motor cortex of adult rats. J Neurosci 19: 1015310163, 1999.
11. Kinouchi H, Huang H, Arai S, Mizoi K, Yoshimoto T. Induction of cyclooxygenase-2 messenger RNA after transient and permanent middle cerebral artery occlusion in rats: comparison with c-fos messenger RNA by using in situ hybridization. J Neurosurg 91: 10051012, 1999.
12. Kittaka M, Giannotta SL, Zelman V, Correale JD, DeGiorgio CM, Weiss MH, Zlokovic BV. Attenuation of brain injury and reduction of neuron-specific enolase by nicardipine in systemic circulation following focal ischemia and reperfusion in a rat model. J Neurosurg 87: 731737, 1997.
13. Klintsova AY, Cowell RM, Swain RA, Napper RM, Goodlett CR, Greenough WT. Therapeutic effects of complex motor training on motor performance deficits induced by neonatal binge-like alcohol exposure in rats. I. Behavioral results. Brain Res 800: 4861, 1998.
14. Koistinaho J, Chan PH.Spreading depression-induced cyclooxygenase-2 expression in the cortex. Neurochem Res 25: 645651, 2000.
15. Markgraf CG, Green EJ, Hurwitz BE, Morikawa E, Dietrich WD, McCabe PM, Ginsberg MD, Schneiderman N. Sensorimotor and cognitive consequences of middle cerebral artery occlusion in rats. Brain Res 575: 238246, 1992.
16. Meyer LS, Kotch LE, Riley EP.Alterations in gait following ethanol exposure during the brain growth spurt in rats. Alcoholism Clin Exp Res 14: 2327, 1990.
17. Nagayama M, Niwa K, Nagayama T, Ross ME, Iadecola C. The cyclooxygenase-2 inhibitor NS-398 ameliorates ischemic brain injury in wild-type mice but not in mice with deletion of the inducible nitric oxide synthase gene. J Cereb Blood Flow Metab 19: 12131219, 1999.
18. National Korean Medical Text Editorial Office. Bon-Cho-Hak (本草學), 1st edn, Seoul: Young-Lim-Sa, pp. 8-15, 2004.
19. Nogawa S, Zhang F, Ross ME, Iadecola C. Cyclo-oxygenase-2 gene expression in Neurons contributes to ischemic brain damage. J Neurosci 17: 27462755, 1997.
20. Rogers DC, Campbell CA, Stretton JL, Mackay KB. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 28: 20602065, 1997.
21. Sibert K, Masferrer J, Zhang Y, Gregory S, Olson G, Hauser S, Leahy K, Perkins W, Isakson P. Mediation of inflammation by cyclooxygenase-2. Agents Actions Suppl 46: 4150, 1995.
22. Stroemer RP, Kent TA, Hulsebosch CE. Neocortical neural sprouting, synaptogenesis, and behavioral recovery after neocortical infarction in rats. Stroke 26: 21352144, 1995.
23. Stroemer RP, Kent TA, Hulsebosch CE. Enhanced neocortical neural sprouting, synaptogenesis, and behavioral recovery with -amphetamine therapy after neocortical infarction in rats. Stroke 29: 23812393, 1998.
24. Yao DL, Masonic K, Petullo D, Li LY, Lincoln C, Wibberley L, Alderson RF, Antonaccio M. Pretreatment with intravenous FGF-13 reduces infarct volume and ameliorates neurological deficits following focal cerebral ischemia in rats. Brain Res 818(1): 140-146, 1999.
25. Yin W, Badr AE, Mychaskiw G, Zhang JH. Down regulation of COX-2 is involved in hyperbaric oxygen treatment in a rat transient focal cerebral ischemia model. Brain Res 926(1-2): 165-171, 2002.
26. Zea Longa EL, Weinstein PR, Carlson S, Summins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20: 84-91, 1989.
27. Colle LM, Holmes LJ, Pappius HM. Correlation between behavioral status and cerebral glucose utilization in rats following freezing lesion. Brain Res 397: 2736, 1986.