Neuroprotective Effect of Palmul-Chongmyeong-Tang on Ischemia-Induced Learning and Memory Deficits in the Rat

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Ginseng Radix, Atractylodis Macrocephalae Rhizoma, Poria, Glycyrrhizae Radix, Angelicae Gigantis Radix, Ligusticum Rhizoma, Rehmanniae Radix, Paeoniae Radix, Acori Graminei Rhizoma, and Polygalae Radix have been widely used as herbal medicine against ischemia. In order to test the neuroprotective effect of a novel prescription, the present study examined the effects of Palmul-Chongmyeong-Tang (PMCMT) consisting of these ten herbs on learning and memory in the Morris water maze task and the central cholinergic system of rats with cerebral ischemia-induced neuronal and cognitive impairments. After middle cerebral artery occlusion (MCAO) for 2 h, rats were administered with saline or PMCMT (200 mg/kg, p.o.) daily for 2 weeks, followed by their training to the tasks. In the water maze test, the animals were trained to find a platform in a fixed position during 6 d and then received a 60 s probe trial on the 7th day following removal of the platform from the pool. Rats with ischemic insults showed impaired learning and memory of the tasks and treatment with PMCMT produced a significant improvement in escape latency to find the platform in the Morris water maze. Consistent with behavioral data, treatment with PMCMT also reduced the loss of cholinergic immunoreactivity in the hippocampus induced by cerebral ischemia. These results demonstrated that PMCMT has a protective effect against ischemia-induced neuronal and cognitive impairments. The present study suggested that PMCMT might be useful in the treatment of vascular dementia.

Key words ischemia; Palmul-Chongmyeong-Tang (PMCMT); learning memory; central cholinergic system; neuroprotection

Improving deficient ability of spatial learning memory has received extensive experimental attention because more and more people lose their learning and memory ability after stroke. Cerebral ischemia is known to produce severe histopathological damage and related behavioral deficits, some of which continue to progress. The middle cerebral artery perfused brain areas such as the parietal cortex, hippocampus, and striatum are mainly affected after cerebral ischemia. In particular, the hippocampal neurons, known to play an important role in learning and memory processes, are vulnerable to neuronal injury produced by ischemia (ISHCH). Lesions of hippocampal cells by ischemia are well established to produce severe deficits of learning and memory in a variety of behavioral tasks.

There have been many studies seeking novel neuroprotective substances. Chinese herbs have been more and more focused on recently. The most frequently studied herbs are Ginseng Radix, Acori Graminei Rhizoma (AGR), Polygalae Radix (PGR), Other frequently used herbs such as Atractylodis Macrocephalae Rhizoma (AMR), Poria, Glycyrrhizae Radix (GR), Angelicae Gigantis Radix (AR), Ligusticum Rhizoma, Rehmanniae Radix (RR), Paeoniae Radix (PR) and other were also investigated eagerly.

Nowadays many experiments are performed using not only one herb or an active compound of an herb but also decoctions-combinations of herbs.

In traditional Oriental medicine, many herbal drugs and prescriptions have been used clinically for the treatment of stroke and vascular dementia. Chongmyeong-tang, a decoction consisting of Acori Graminei Rhizoma, Polygalae Radix and Poria has been known to induce resuscitation and have an effect on amnesia. Palmultang is another famous prescription composed of Ginseng Radix, Atractylodis Macrocephalae Rhizoma, Poria, Glycyrrhizae Radix, Angelicae Gigantis Radix, Ligusticum Rhizoma, Rehmanniae Radix and Paeoniae Radix.

The purpose of this study was to examine the effect of Palmul-Chongmyeong-Tang (PMCMT) composed of ten neuroprotective herbs on impairment of learning and memory in ischemia-induced amnesia rats using the Morris water maze. The neuroprotective effects of these herbal drugs on the central acetylcholine system were also examined through the histochemistry of hippocampal neurons.

MATERIALS AND METHODS

Animals Adult male Sprague-Dawley rats weighing 250—280 g were obtained from Samtaco Animal Corp. (Seoul, Korea). All animals were housed in groups of five or six with continuous access to food and water ad libitum and were maintained on a 12 h light/dark cycle regulated at 23 °C room temperature. The experiments began at least 7 d after their arrival in individual home cages. Animal experiments were carried out in accordance with the Prevention of Cruelty to Animals Act 1986 and NIH guidance for the care and use of laboratory animals for experimental procedures, and were approved by local committee review.

Preparation of PMCMT All herbs composing PMCMT were purchased from an oriental drug store (Jungdo, Inc.,
duced using the intraluminal filament technique. Anesthesia was induced using 3% isoflurane in 30% O₂/70% N₂O and maintained throughout the operation with 0.5 to 0.6% isoflurane delivered via a nose mask. The right common carotid artery was exposed through a midline cervical incision. The heparinized intraluminal filament (ϕ 0.28 mm, rounded tip) was introduced via the external carotid artery. The rectal temperature was monitored and maintained at 37°C using a heating pad (Harvard Homeothermic Blanket Control Unit, 50-7061). After 120 min of the occlusion, the filament was gently pulled out and the external carotid artery was permanently closed by cautery. In sham-operated rats, the right common carotid artery was exposed and the external carotid artery was opened without introducing the filament into the internal carotid artery. After the operation, the animals were allowed to wake up in an incubator (30 °C) and were then moved to their home cages.

Experimental Groups

PMCMT was dissolved in 0.9% NaCl and rats were divided into three groups. The experimental group was treated with PMCMT (200 mg/kg, PMCMT+ISCH group (n=8)) for 2 weeks after induction of ischemia. The control group was treated with saline (0.1 mg/kg, SAL+ISCH group (n=7)) for 2 weeks after induction of ischemia. PMCMT and saline were administrated orally every day. The sham-operated control group (SHAM, n=6) was not treated with any drug for 2 weeks after induction of ischemia. The water maze tests were performed in the 3rd week after ischemia had been induced.

Water Maze Task

The water maze was a circular pool (painted white, 2.0 m in diameter, 0.35 m high) constructed of fiberglass. The water contained was maintained at a temperature of 22±2°C, and was made opaque by the addition of 1 kg of powdered skim milk. Testing in the water maze a platform 15 cm in diameter, was located 1.5 cm below the water surface in one of four locations in the pool, approximately 50 cm from the side walls. The pool was surrounded by many cues external to the maze. A video camera was mounted in the ceiling above the pool and was connected to a video-recorder and tracking device (S-MART; Pan-Lab, Barcelona, Spain), which permitted on- and off-line automated tracking of the path taken by the rat. The animals were subjected to four trials per session. The rats were trained to locate the hidden escape platform, which remained in a fixed location throughout the testing. Trials lasted a maximum of 180 s and the latency and swim distance to find the submerged platform were recorded. The animals were tested in this way for 6 d, and then were given a probe trial on the 7th day. For the probe trial, the platform was removed from the pool and the animal was released from the quadrant opposite where the platform had been located. The length of the trial was 60 s, after which the rat was taken out of the pool. The proportion of time and swim distance the rat spent searching for the platform in the training quadrant, i.e., the previous location of the platform, were recorded and used as a measure of retention. In this study, swimming time and distance within only a 30 cm circular zone around the previous platform, that is, not in the whole quadrant, were recorded.

Cholineacetyltransferase (ChAT) Immunohistochemistry

At the end of the behavioral observation, rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and then perfused through the ascending aorta with normal saline (0.9%), followed by 900 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, postfixed overnight, and cryoprotected in 20% sucrose with PBS. Brains were cut by a cryostat into 30-μm coronal sections, which were processed immunohistochemically as free-floating sections.

The sections were washed in PBS containing 0.3% Triton X-100 and 1% rabbit serum and were then incubated in the ChAT primary antibody (Cambridge Research Biochemicals, Wilmington, DE, U.S.A.) diluted 1:2000 in the same buffer at 4 °C for 72 h. After washing, the sections were incubated in biotinylated anti-sheep serum and ABC complex (Vectorstain Elite Kit; Vector Lab., Burlingame, CA, U.S.A.) for 2 h. The ABC complex was visualized with 0.5% diaminobenzidine with 0.02% H₂O₂. Images were captured using an Axio Vision 3.0 imaging system (Zeiss, Oberkochen, Germany) and processed in Adobe Photoshop. To measure cells of ChAT, the grid was placed on the hippocampal CA1 area according to the atlas of Paxinos and Watson. Number of cells was counted at 100× magnification using a rectangular microscope grid measuring 100×100 μm.

ACH E Histochemistry

The sections were washed in PBS and incubated in a solution with 25 mg acetylthiocholine iodine for 1 h. The solution was composed of 32.5 ml 0.1 M sodium hydrogen phosphate buffer (NaH₂PO₄, H₂O, pH 6.0), 2.5 ml 0.1 M sodium citrate, 5 ml 30 mM copper sulfate, 5 ml 5 mM potassium ferricyanide, and 5 ml distilled water. Color of the mixing solution was a pretty green. The density of stained nuclei of hippocampal cells was measured using the Scion image program (Scion Corp., Frederick, MD, U.S.A.).

Statistical Analysis

The data were expressed as means±S.E. Group differences in the escape latency in the Morris water maze task were analyzed using one-way analysis of variance (ANOVA) with repeated measures. One-way ANOVA followed by the Tukey post hoc test multiple group comparison was used to analyze group differences of the data collected during successive training days, probe trials, immunohistochemical assay, and image analysis. A difference between groups was considered as statistically reliable if the associated probability (p-value) was below 0.05.

RESULTS

Effect of PMCMT on the Water Maze Test

The forebrain ischemia affected the performance of the rats in the water maze. The SAL+ISCH group showed worse performance than the SHAM group with their latencies in finding the hidden platform being significantly increased, as seen in Fig. 1.
Four trials per day over 6 d were performed for the acquisition test. Rats were treated with PMCMT (200 mg/kg, p.o., PMCMT+ISCH, n=8) for 2 weeks after induction of cerebral ischemia. The sham-operated control group (SHAM, n=6) and the ischemia group (SAL+ISCH, n=7) were not given any drug for 2 weeks after cerebral ischemia. Significance with Tukey’s test following a repeated ANOVA is indicated as ∗p<0.05, ∗∗p<0.01, ∗∗∗p<0.001 (Sham vs. SAL+ISCH), or ∗p<0.05 (SAL+ISCH vs. PMCMT+ISCH). Vertical lines indicate S.E.M. (n=6—8).

The task was performed with four daily trials on the 7th day without the platform for the retention test. Rats were treated with PMCMT (200 mg/kg, p.o., PMCMT+ISCH, n=8) for 2 weeks after cerebral ischemia. The sham-operated control group (SHAM, n=6) and the ischemia group (SAL+ISCH, n=7) were not given any drug for 2 weeks after induction of ischemia. Significance with Tukey’s test following a repeated ANOVA is indicated as ∗∗∗p<0.001, ∗∗p<0.01, ∗p<0.05 (Sham vs. SAL+ISCH), or ∗p<0.05 (SAL+ISCH vs. PMCMT+ISCH). Vertical lines indicate S.E.M. (n=6—8).

An ANOVA (4×6, treatment×time) performed on the swimming time of acquisition trials revealed significant effect of a group difference (F(2,18)=9.268, p<0.01), effect of day (F(5,90)=36.901, p<0.001), but not group day interaction (F(10,90)=0.594, p=0.815). Tukey’s post-hoc test revealed that the PMCMT+ISCH group showed significantly reduced latency of swimming time, compared with those of the SAL+ISCH group (p<0.05 on day 5 and 6, respectively) (Fig. 1). On the 7th day, post-hoc test on retention performance also revealed that the PMCMT+ISCH group spent longer time around the platform than the ISCH group (p<0.05) (Fig. 2).

An ANOVA (4×6, treatment×time) performed on the swimming distance of acquisition trials revealed also significant effect of a group difference (F(2,18)=10.082, p<0.01), effect of day (F(5,90)=41.928, p<0.001), but not group day interaction (F(10,90)=0.485, p=0.896). Tukey’s post-hoc test revealed that PMCMT+ISCH group showed significantly reduced swimming distance than the SAL+ISCH group (p<0.05 on day 5) (Fig. 3). On the 7th day, the post-hoc test on retention performance also revealed that the PMCMT+ISCH group spent longer time around the platform than the ISCH group (p<0.05) (Fig. 4). Ischemia severely impaired spatial cognition in the water maze task, but administration of PMCMT (200 mg/kg) attenuated ischemia-induced learning and memory damage in this task.

Effect of PMCMT on the Central Cholinergic System
ChAT Immunohistochemistry The results of the ChAT immunoreactive analysis in the CA1 are shown in Figs. 3 and 4. The number of ChAT-immunoreactive neurons was 29.22±1.94 (100.0±0.0%) in the sham group, 24.28±0.90 (83.08±3.07%) in the SAL+ISCH group, and 26.63±2.02 (91.11±6.92%) in the PMCMT+ISCH group [(2,63)=8.446, p<0.01]. The Tukey post-hoc test revealed that the number of ChAT neurons significantly increased in the PMCMT+ISCH group compared to the SAL+ISCH group (p<0.05 in the CA1 area) (Figs. 5, 6).

Acetylcholinesterase (AChE) Histochemistry The density of AChE fibers in the hippocampal formation was lower in the SAL+ISCH group than the SHAM group, as shown in Figs. 7 and 8. The density of AChE neurons in the CA1 area was 123.28±2.14 (100.0±0.0%) in the SHAM group, 111.29±2.26 (90.27±1.83%) in the SAL+ISCH group, and 118.13±4.17 (95.82±3.39%) in the PMCMT+ISCH group.
The Tukey post-hoc test revealed that the density of AChE reactive neurons in the hippocampus of the PMCMT ISCH group was greater than that of the SAL ISCH group ($p = 0.239$ in the CA1).

**DISCUSSION**

The present study demonstrated that focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO) produced severe deficits in rat performance in a Morris water maze along with signs of neurodegeneration, including decreased ChAT and AChE activity in the hippocampus. Treatment with PMCMT attenuated ischemia-induced learning and memory deficits in the maze and had a protective effect against ischemia-induced decrease in cholinergic neurons.

The extent of brain damage produced by MCAO is known to be dependent on the degree of the ischemic insult and its duration. One of the most critical factors is the period of occlusion. The damage produced by mild injury (30 min of MCAO) was confined to the striatum or cortex, whereas more than 2 h exposure to MCAO led to damage of the striatum, cortex and more remote areas including the hippocampus.\(^6\) Of the neighboring regions undergoing delayed cell death in response to ischemic insults, the most vulnerable are found in the hippocampus, which plays a major role in learning and memory.\(^1\) In the present study the ischemia group showed significant cell loss of the striatum and the parietal cortex as well as hippocampal CA1 area (data not shown). However, the reduction of neuronal damage in the hippocam-
The Morris water maze task is thought to test permanent spatial learning capability and reference memory. Similar to the previous studies, ischemic animals exhibited mean speed, movement time, and rest time that were not significantly different from sham animals, indicating that any abnormalities revealed by this task could not be due to motor impairment. The motor deficits were not obvious in either the nontreated or treated groups by casual observation at the time of the water maze task. Many researchers also thought that if there remained mild motor dysfunction, it was unlikely that it accounted for the deficiency in water maze test. The water extract of Poria was reported to promote hippocampal long-term potentiation in vivo. A traditional Chinese medicinal formula including Poria reduced the production of nitric oxide in cortical tissue after repeated ischemia-reperfusion and significantly improved cognitive function by elongating latency and reducing the number of errors in the step-through test. Acor Korea (AGR) and Polygalae Radix (PGR) are well-known major oriental medicines which have long been included in medical prescriptions for the treatment of stroke and vascular dementia. Recently, several studies have indicated ameliorative effects of AGR on learning and memory impairments. AGR protected ischemia-induced neuronal death and cognitive impairments in the rat. AGR and its major component, asarone, also have a neuroprotective effect against excitotoxic neural death and the effect may be through the blockade of NMDA receptor. PGR extract was shown to protect cultured rat granule cells against damage induced by NMDA and has some reparative effects on the memory and behavioral disorders produced by lesioning of the nucleus basalis magnocellularis (NBM) in rats. Some oriental prescriptions including AGR, PGR, and Ginseng (Chongmyeong-Tang consists of these three herbs), and Ginseng Radix have been shown to improve performance on avoidance learning and memory tasks.

Aqueous extract of Glycyrrhizae Radix (GR; licorice) significantly improved learning and memory of mice and reversed the amnesia induced by diazepam, scopolamine and ethanol. Atractyloides Macrocephalae Rhizoma (AMR) improved scopolamine-induced impairment of spatial memory and a prescription including AMR also ameliorated cognitive dysfunction in a murine model for vascular dementia. Angelicae Gigantis Radix (AR) has mainly been studied in relation to its inhibitory effect on blood coagulation. Tang-gui-shao-yao-san, a famous prescription including AR, Ligusticum, Poria and AMR improved the scopolamine-induced impairment of rat spatial cognition in an eight-armed radial maze test and the AR fraction extracted by the butanol layer was suggested to play an important role. The same prescription also prevented the impairment of spatial memory induced by repeated cerebral ischemia in rats. Paeoniae Radix (PR) suppressed sodium current in acutely dissociated rat hippocampal CA1 neurons, which may indicate protective effect of PR during brain ischemia. PR extract also showed a protective effect against nitric oxide donor-induced neuronal death in cultured cerebellar granule cells. Ligusticum Rhizoma is another Chinese herb well known for its strong activity on blood circulation and many studies have been made to prove its protective effect in ischemic stroke since the late 1980s. Recent studies revealed more about the mechanism of its effect: it decreased the infarct size and behavior deficits score in the MCAO model, and its inhibition of platelet-dependent thrombosis and amelioration of hemorheological parameters are proposed as its protective mechanism. Rehmanniae Radix (RR) improved the function of learning and memory in rats with damaged thalamic arcuate nucleus, and the mechanism might be related to the increased expression of hippocampal c-fos and NGF. A traditional Korean formulation including RR, Ginseng, AGR and PGR showed an attenuating effect on H2O2-induced toxicity in PC12 cells.

Nowadays studies on neuroprotective herbs are showing a diverse tendency. Research is more and more focused on finding a single effective compound of one herb, rather than the whole herb or identifying the exact mechanism of action. Other studies focus on the novel effect of a combination of herbs (prescription or decoction). Herb constituents may not only act synergistically with other constituents from the same herb but may also enhance the activity of or counteract toxic effects of compounds from other herb species. Many traditional Korean herbal drugs are known to have a synergistic effect with others; this effect can be explained by various action mechanisms of each herb. A new prescription consisting of Korean red ginseng and four other herbs was found by Yun et al. to be more effective than the red ginseng alone on antithrombotic activity. This study, the neuroprotective effect of PMCMT could not be explained by the action of one or two herbs, for the dose of each of the ten herbs was much less than used in other studies. The dose of 200 mg/kg of PMCMT indicates approximately 20 mg/kg of each of the ten herbs, 1/5—1/10 the dose used in other studies. For example, 350 mg/kg of ginseng extract was given orally and 500 mg/kg or even 1000 mg/kg was administered to prove its neuroprotective effect. PGR extract was administered at a dose of 100 mg/kg or 250 mg/kg. The dosage (200 mg/kg) of PMCMT chosen in the present study is a relatively standard dose of natural herbal medicine reported by other workers in rodent experiments. The dose of 200 mg/kg was also close to the clinically prescribed dose for human. Therefore, we also selected one single dose and examined its long-term behavioral effects in the present study, in order to screen the candidate for its potential as a cognitive enhancing drug. The dose—response in toxicity and effect of PMCMT should be investigated in the near future.

In many studies the neuroprotective effect showed dose-dependency, but higher dose did not always exert a more potent effect. Memory-strengthening activity of licorice was studied in exteroceptive and interoceptive behavioral models, and three doses (75, 150, 300 mg/kg) of an aqueous extract of licorice were administered to separate groups of...
mice. The dose of 150 mg/kg significantly improved learning and memory of the animals. A Chinese medicine including Chunaxiong and Poria (0.03 g/kg, 0.3 g/kg, 1 g/kg, 3 g/kg a day) greatly improved the blood flow in rats with cerebral ischemia, and 0.3 g/kg increased cerebral blood flow to the normal level.30) The present study attempted to clarify the effect of Palmul-Chongmyeong-Tang (PMCMT) composed of ten neuroprotective herbs on ischemia-induced impairment of learning and memory using the Morris water maze. The neuroprotective effects of these herbal drugs on the central acetylcholine system were also examined by histochemistry of hippocampal neurons. PMCMT is a new prescription consisting of two old prescriptions, Palmul-Tang and Chongmyeong-Tang. In Dong-Eu-Bo-Gam (an old Korean traditional medicine book compiled by Hu Jun), Chongmyeong-Tang (a decoction consisting of Acori Graminei Rhizome, Polygalae Radix and Poria) reportedly induced resuscitation and had an effect on amnesia. Palmul-tang is another famous prescription composed of Sagunja-Tang (it has the effect of reinforcing Qi and is composed of Ginseng Radix, AMR, Poria and GR) and Samul-Tang (which reinforces blood activity and is made up of Angelicae Gigantis Radix, Ligusticum Rhizoma, RR and PR). The neuroprotective effect of PMCMT can be anticipated on the basis of the traditional Korean medicine theory of Palmul-Tang and Chongmyeong-Tang as well as many studies on each of the ten herbs. The forebrain ischemia affected the performance of rats in the water maze. The escape latencies of the ischemia group were significantly increased and they spent a shorter time around the platform compared to the sham-operated group. Moreover, the number of ChAT neurons and the density of AChE fibers in the hippocampus were significantly decreased. In summary, treatment with PMCMT attenuated ischemia-induced learning and memory deficits in the Morris water maze and had a protective effect against ischemia-induced decrease in cholinergic neurons. PMCMT is thus a good candidate for further investigations that may ultimately result in its clinical use.

Further study is required to determine more effective herbal combinations and to find the most appropriate administrational dose. Acori Graminei Rhizome, Polygalae Radix and Ginseng Radix are thought to have played an important role in PMCMT in this study, but the synergistic effect of other herbs should also be studied. A study design using different combinations and different proportions of the ten herbs would be helpful. Clinical efficacy and potential toxicity of active plants and compounds in larger trials require further assessment before recommendations regarding their use can be established. Many other herbs traditionally used for the treatment of stroke also need to be evaluated as component candidates of novel prescriptions for cerebral ischemia.

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