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New Four-herb Formula Ameliorates Memory Impairments via Neuroprotective Effects on Hippocampal Cells

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The current study was conducted to evaluate beneficial effects of a new formula (CWC-9) using four traditional Oriental medicinal herbs, *Cynanchum wilfordii*, *Rehmannia glutinosa*, *Polygala tenuifolia*, and *Acorus gramineus*, on hippocampal cells and memory function. To examine the neuroprotective effects of a new four-herb extract, cell viability, cytotoxicity, and reactive oxygen species (ROS) assays were performed in HT22 cells and behavioral tests (Morris water maze and passive avoidance retention), Western blot, and immunohistochemistry were performed in a mouse model of focal cerebral ischemia. In HT22 hippocampal cells, pretreatment with CWC-9 resulted in significantly reduced glutamate-induced cell death with suppression of ROS accumulation caused by glutamate. In a mouse model of focal cerebral ischemia, we observed significant improvement of spatial and short-term memory function by treatment with CWC-9. Phosphorylated p38 mitogen-activated protein kinases (MAPK) in hippocampus of ischemic mice were decreased by treatment with CWC-9, but phosphorylated phosphatidylinositol-3 kinase (PI3K) and cAMP response element binding protein (CREB) were significantly enhanced. By immunohistochemical analysis, we confirmed higher expression of phosphorylation of CREB in the hippocampal neurons of CWC-9 treated mice. These results suggest that new multi-herb formula CWC-9 mainly exerted beneficial effects on cognitive function through regulation of neuroprotective signaling pathways associated with CREB.

Key words : Focal cerebral ischemia, hippocampal neurons, memory, neuroprotection

Introduction

As an aging population is occurring on a global scale, the incidence of memory and cognitive impairments including stroke is likely to show a significant increase in the near future [12]. Damage of hippocampal neurons can explain the progress of learning and memory impairment in a wide number of brain disorders [6, 16]. Effective new therapeutic strategies are needed to prevent hippocampal neuronal deaths, which can ameliorate cognitive dysfunction of many neurological disorders.

The neuroprotective function has been verified in HT22 hippocampal cells and middle cerebral artery occlusion (MCAO)-induced brain injury [10, 13]. Neuronal death is the result of excessive oxidative stress in a variety of neurodegenerative disorders of the central nervous system [5, 26]. Prolonged exposure to high concentrations of glutamate shows reactive oxygen species (ROS)-mediated oxidative toxicity in HT22 and cerebral ischemic models [8, 24].

Neurons contain low levels of endogenous antioxidant [5], consequently, the brain is unusually vulnerable to ROS mediated neuronal cell death and cerebral ischemic injury [18, 25]. Functional deficits associated with ischemic neuronal damage in MCAO models are regulated by an antioxidative mechanism [13]. Activation of p38 mitogen-activated protein kinase (MAPK) is generally implicated in neuronal death, while the phosphatidylinositol-3 kinase (PI3K) pathways lead to cell survival against oxidative stress [22]. Expression of cyclic AMP response element binding protein (CREB) in different brain areas plays a role in neuronal plasticity, memory, and learning as well as promoting neuronal survival in the brain [14].

After systematic literature investigation of Donguibogam, published by Joon Hur in the early 17th century in Korean, we prepared a multi-herb extract composed of *Cynanchum*

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wilfordii Hemsley, *Rehmannia glutinosa* (Gaertn) Libosch., *Polygala tenuifolia* Willd., and *Acorus gramineus* Soland. to demonstrate the synergistic effects. Approach to prevention and treatment using a multi-herb formula may be more effective in age-related dementia and memory impairment [20]. A new multi-herb extract may be a potential candidate for protection of hippocampal neuronal cells with subsequent improvement of learning and memory impairment. Therefore, we investigated the beneficial effects of herbal mixture extract on hippocampal neurons in HT22 cells and hippocampus of focal cerebral ischemia mice.

Material and Methods

Chemicals and antibodies

L-glutamate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture reagents were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). Antibodies recognizing p38 and phospho-p38 (pp38, Thr180/Tyr182) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA), and PI3K, phospho-PI3K (pPI3K, Tyr458), CREB, and phospho-CREB (pCREB, Ser133) were supplied by Cell Signaling (Danvers, MA, USA). Antibody recognizing neuronal nuclei (NeuN) was supplied by Millipore Corporation (Billerica, MA, USA). Secondary antibodies were supplied by ENZO Life Sciences (Farmingdale, NY, USA). A lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Promega (Madison, WI, USA), and ROS detection reagent, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), was purchased from Invitrogen (Carlsbad, CA, USA).

Preparation of four herbal mixture extract

The dried roots of *Cynanchum wilfordii* Hemsley, *Rehmannia glutinosa* (Gaertn) Libosch., *Polygala tenuifolia* Willd., and *Acorus gramineus* Soland. were purchased from Dongnam Co. (Busan, Korea) and were authenticated by Professor Y.W. Choi, Department of Horticultural Bioscience, College of Natural Resources and Life Science, Pusan National University. A voucher specimen (accession number CWCWSD2; CWC-9) was deposited at the Plant Drug Research Laboratory of Pusan National University (Miryang, Korea). Dried powdered *Cynanchum wilfordii* (25.5 kg), *Rehmannia glutinosa* (9.5 kg), *Polygala tenuifolia* (7.5 kg), and *Acorus gramineus* roots (7.5 kg) were immersed in 450 L of distilled water and boiled at 120±5℃ for 150 min. The resultant extract was centrifuged (2,000× *g* for 20 min at 4℃) and filtered through a 0.2-μm filter. The filtrate was then concentrated in vacuo at 70±5℃ under reduced pressure and then converted into a fine spray-dried powder at a yielding rate of 6.0% (3.0 kg) in a vacuum drying apparatus. Finally, the solid form of the spray-dried powder was dissolved with dimethyl sulfoxide (DMSO) for use as CWC-9 in experiments.

Cell culture

HT22 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% CO_2 humidified incubator at 37℃. The cells were incubated for 24 hr prior to experimental treatments. After incubation, cells were treated with various concentrations of CWC-9 for 24 hr, followed by exposure to 5 mM glutamate for 24 hr. CWC-9 was removed from the medium during exposure to glutamate.

Determination of cell viability and cytotoxicity

For MTT assay, cells were incubated with 0.5 mg/ml MTT in culture medium for 4 hr at 37℃ and absorbance was read at 595 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as a percentage of controls. Release of LDH from damaged cells was performed according to the manufacturer's instructions for the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega). Absorbance was measured using a SpectraMax 190 spectrophotometer (Molecular Devices) at 490 nm. Data represent the percentage of LDH released relative to the assay positive controls.

ROS measurement

HT22 cells were cultured in 96-well white plates at a density of 5×10^3 cells per well. After adherence, cells were pretreated with CWC-9 for 24 hr and then exposed to 5 mM glutamate for 24 hr. Treated cells were washed with PBS. Carboxy-H_2DCFDA (20 μM) (Invitrogen) was applied to the cells, followed by incubation for 1 hr in a 37℃ incubator. Fluorescence was measured using a Mutilabel counter (Perkin Elmer 1420, MA, USA).

Focal cerebral ischemia and CWC-9 administration

To confirm beneficial effects of CWC-9 on hippocampal cells, we used middle cerebral artery occlusion (MCAO) model. Male C57BL/6 mice (20-25 g) were obtained from Dooyeol Biotech (Seoul, Korea). The mice were housed at 22℃ under alternating 12 hr cycles of dark and light, and were fed a commercial diet and allowed tap water *ad libitum*. All experiments were approved by the Pusan National University Animal Care and Use Committee (Approval number, PNU 2014-0517). Each group consisted of six mice and all treatments were administered under isoflurane (Choongwae, Seoul, Korea) anesthesia, which was provided using a calibrated vaporizer (Midmark VIP3000, Orchad Park, OH, USA).

Focal cerebral ischemia was induced by occluding the middle cerebral artery using the intraluminal filament technique. A fiber-optic probe was affixed to the skull over the middle cerebral artery for measurement of regional cerebral blood flow using a PeriFlux Laser Doppler System 5000 (Perimed, Stockholm, Sweden). Middle cerebral artery occlusion model was induced by a silicon-coated 4-0 monofilament in the internal carotid artery and the monofilament was advanced to occlude the middle cerebral artery. The filament was withdrawn 30 min after occlusion and reperfusion was confirmed using laser Doppler. Mice in the CWC-9 groups received oral administration daily at the doses of 100 and 500 mg/kg for three weeks after MCAO, while mice in the control and vehicle groups were only given distilled water at the same intervals.

Behavioral assessment

In experiment 1, acquisition training for the Morris water maze was performed on four consecutive days from 10 days to 7 days before MCAO (5 trials per day) and basal time was measured at 6 days before MCAO. The tank had a diameter of 100 cm and an altitude of 50 cm. The platform was placed 0.5 cm beneath the surface of the water. Each trial was performed for 90 s or until the mouse arrived on the platform. After final administration of CWC-9, results of the experiment were recorded using SMART 2.5.18 (Panlab S.L.U.).

In experiment 2, a passive avoidance test was performed in an apparatus containing an illuminated (light) and a dark compartment separated by an automatic guillotine door (Med-Associates, Inc., Albans, VT). During acquisition trials, once the mouse crossed into the dark compartment, it received a 0.5 mA electric foot shock for 3 s. Approximately 24 hr later, a retention trial was administered by placing the mouse in the light compartment and recording the step-through latency, during which time no shocks were administered. Maximal testing time was 600 s. Animals who failed to enter the dark compartment within 600 s were assigned a maximum test latency score of 600 s. Results of the experiment were recorded using MED-PC software interfaced to chambers.

Western blot analysis

Tissue punches from each hippocampus were sonicated in ice cold lysis buffer [200 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1 mM NaF, 1% NP40, 1 mM PMSF, 1 mM Na_3VO_4, protease inhibitor cocktail]. Equal amounts of proteins were separated by 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membranes were blocked with 5% skim milk in PBST for 1 h, followed by overnight exposure to appropriate antibodies. Membranes were then incubated with appropriate horseradish peroxidase-conjugated antibodies for 1 hr. All specific bands were visualized using an enhanced chemiluminescence system (Pierce Biotech, Rockford, IL, USA) and imaged using an Image Quant LAS-4000 imaging system (GE Healthcare Life Science, Uppsala, Sweden). Results of the Western blot assay reported here are representative of at least three experiments.

Immunofluorescence staining

Mice anesthetized with isoflurane received intracardial perfusion with saline and then 4% paraformaldehyde in PBS. Brains were removed, post-fixed in the same fixative for 4 hr at 4℃, and immersed in 30% sucrose for 48 hr at 4℃ for cryoprotection. Frozen 20 μm-thick sections were incubated for blocking with a blocking buffer (1X PBS/5% normal serum/0.3% Triton X-100) for 1 hr at room temperature. The sections were incubated with the following primary antibodies to NeuN (Millipore Corporation), and pCREB (Cell Signaling) for 20 hr at 4℃. After washes with PBS, the sections were incubated with the fluorescent secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA) for 2 hr at room temperature in the dark, respectively, and then washed three times with PBS. Subsequently, slides were mounted in the mounting medium (Vector Laboratories, Inc.) and captured using a fluorescence microscope.

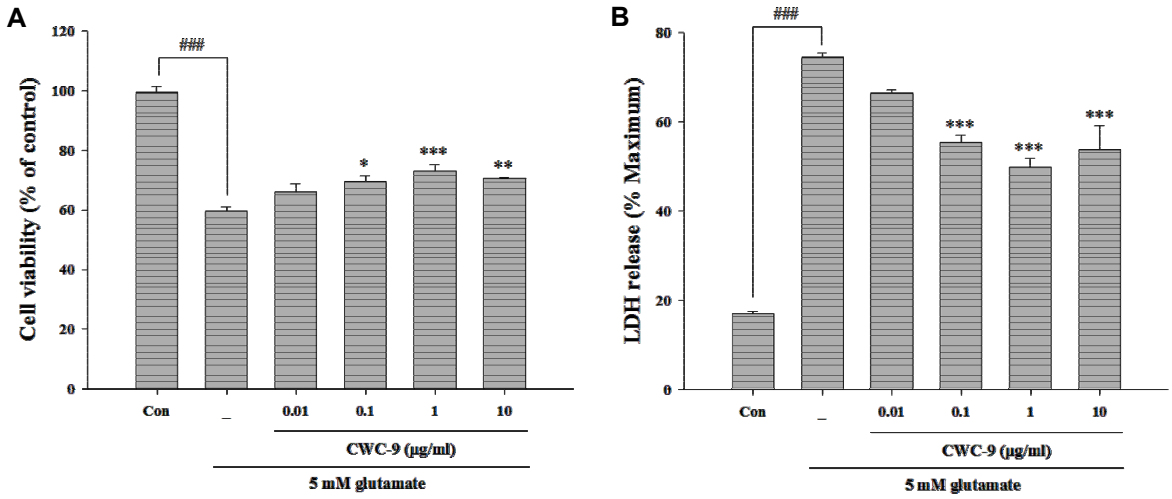

Fig. 1. Effect of CWC-9 on glutamate-induced cell death in HT22 cells. Cell viability and toxicity were determined by MTT (A) and LDH assay (B). Cells were pretreated with 0.01, 0.1, 1, and 10 μg/ml of CWC-9 for 24 hr, followed by exposure to 5 mM glutamate for 24 hr. $^{###}p<0.001$ vs. control; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. glutamate-treated cells. All data are represented as the mean±SEM of three independent experiments.

Data analysis

All data were expressed as mean±SEM and analyzed using the SigmaStat statistical program version 11.2 (Systat Software, San Jose, CA, USA). Statistical comparisons were performed using one-way analysis of variance (ANOVA) for repeated measures followed by Tukey's test of least significant difference. A *P*-value<0.05 was considered to indicate a statistically significant result.

Results

Pretreatment with CWC-9 protects hippocampal cells from oxidative glutamate toxicity

To assess the neuroprotective effect of CWC-9 under conditions of glutamate toxicity, HT22 was challenged with 5 mM glutamate with and without pretreatment of CWC-9. Cell viability in the glutamate treated group declined by approximately 40.4% compared with the control. Pretreatment of cells with CWC-9 at a concentration range of 0.1 to 10 μg/ml resulted in reduced glutamate-induced cytotoxicity (Fig. 1A). The levels of LDH release showed a significant increase to 74.5% after exposure to glutamate, while pretreatment with CWC-9 resulted in a marked decrease of glutamate-induced release of LDH (Fig. 1B).

Because oxidative stress mediates glutamate-induced cell death in HT22 cells via ROS accumulation, we examined the effect of CWC-9 on oxidative stress promoted by glutamate toxicity. Pretreatment with CWC-9 resulted in a significant decrease in ROS production, which prevented elevation of ROS level caused by exposure to glutamate (Fig. 2). These results suggest that pretreatment with CWC-9 exerts a potent neuroprotective effect against oxidative toxicity caused by exposure to glutamate in HT22 hippocampal cells.

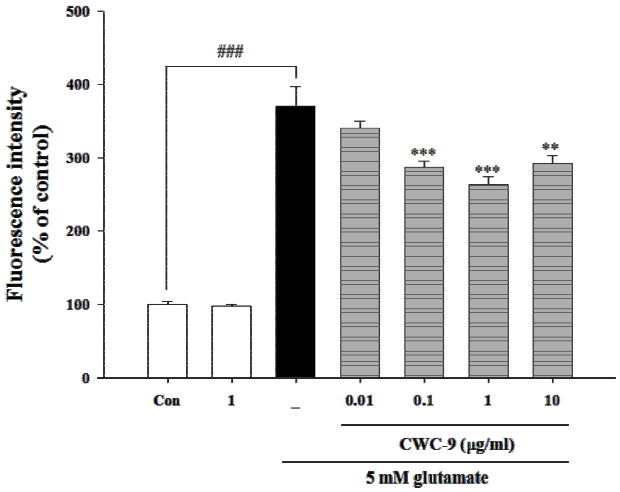

Fig. 2. Effect of CWC-9 on ROS generation in glutamate-treated HT22 cells. Cells were pretreated with 0.01, 0.1, 1, or 10 μg/ml of CWC-9 for 24 hr, followed by exposure to 5 mM glutamate for 24 hr. The oxidation sensitive fluorescence dye, carboxy-H_2DCFDA (20 μM), was used in measurement of ROS levels. Production of ROS was analyzed using a fluorescence plate reader. $^{###}p<0.001$ vs. control; $^{**}p<0.01$ and $^{***}p<0.001$ vs. glutamate-treated cells. All data are represented as the mean±SEM of three independent experiments.

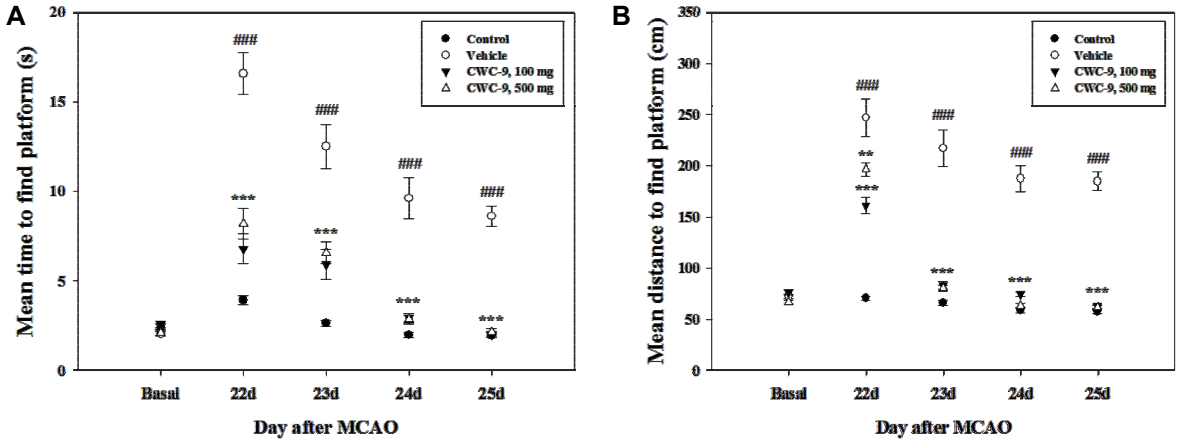

Fig. 3. Beneficial effects of CWC-9 on the spatial memory function in MCAO mice. Morris water maze test was performed from 22 d to 25 d after MCAO. Administration of CWC-9 resulted in significantly improved memory function of MCAO mice during the late phase of the experiment. Mean±SEM. ### $p<0.001$ vs. control; ** $p<0.01$ and *** $p<0.001$ vs. MCAO mice (vehicle).

Treatment with CWC-9 ameliorates memory impairment in MCAO mice

Spatial memory was assessed using the water maze test. MCAO mice took a longer time and distance on average to find the platform than the basal group. However, CWC-9-administered mice attained a significantly lower time and total distance at both concentrations from 22 to 25 days after MCAO compared to the vehicle group (Fig. 3). We assessed whether CWC-9 improves short-term memory in the passive avoidance task. A significant group effect was observed in the step-through latency in the retention trial. The MCAO group exhibited a decreased step-through latency time compared with the control group. However, treatment with CWC-9 at a dose of 100 or 500 mg/kg resulted in significantly prolonged step-through latency compared with the MCAO group (Fig. 4). During the acquisition trials, no significant inter-group differences in step-through latency were observed. These results suggest that treatment with CWC-9 may induce beneficial effects for improvement of memory function in a focal cerebral ischemia model.

Treatment with CWC-9 enhances CREB phosphorylation via signaling pathway of p38 MAPK and PI3K in the hippocampus of MCAO mice

Western blot analysis was performed to determine whether CWC-9 could modulate glutamate-mediated cell death or survival signaling. Protein levels of p38 MAPK phosphorylation were significantly decreased by treatment with CWC-9 compared to the MCAO group, while the levels of phosphorylated PI3K and CREB phosphorylation were increased by treatment with CWC-9 in a dose-dependent manner (Fig. 5). Positive neuronal cells of pCREB/NeuN in the hippocampal CA1 and dentate gyrus (DG) regions were counted after double-label immunofluorescence staining at 26 days after MCAO. Treatment with CWC-9 followed by MCAO surgery resulted in a significant increase in the num-

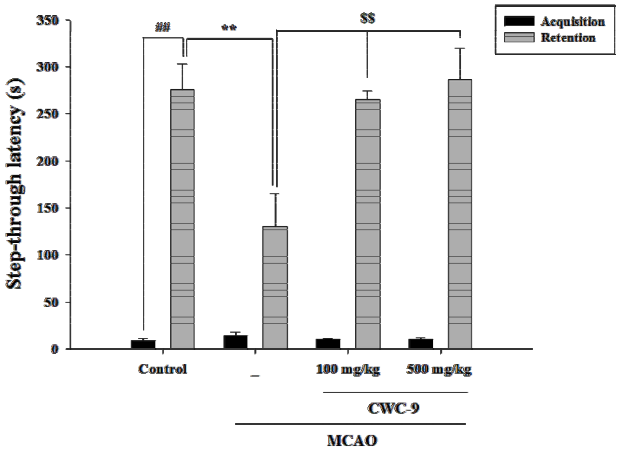

Fig. 4. Beneficial effects of CWC-9 on the short-term memory function in MCAO mice. Retention of the passive avoidance task was assessed 24 days after MCAO. The retention test was preceded by a training session on days 22(habituation) and 23(acquisition). Data are presented as the latency to cross into a compartment in which the mice have previously received an electric foot-shock. No shock was applied during the testing phase, and the maximal testing time was 600 s. Administration of CWC-9 resulted in significantly improved short-term memory function of MCAO mice. Mean±SEM. ## $p<0.01$ vs. control (acquisition); ** $p<0.01$ vs. control (retention); $^{\$\$}p<0.01$ vs. MCAO mice (retention).

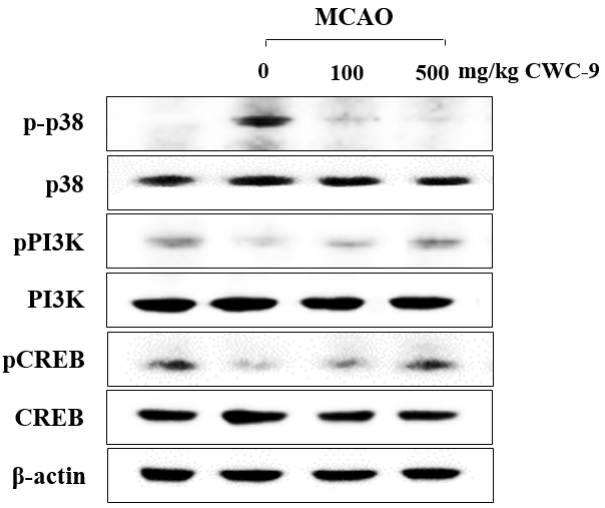

Fig. 5. Effect of CWC-9 on expression of intracellular kinases in the hippocampus at 26 days after MCAO. Equal amounts of proteins and each sample were subjected to Western blot analysis using the indicated antibodies. Equal protein loading was confirmed by β-actin expression.

ber of pCREB/NeuN double-positive cells in the CA1 and DG region of the ipsilateral hippocampus compared to the MCAO group (Fig. 6). These results suggest that CWC-9 may prevent hippocampal neuronal death via both p38 MAPK and PI3K signaling with enhanced phosphorylation of CREB in MCAO mice.

Discussion

Here we investigated the neuroprotective effects of a new herbal mixture extract on hippocampal neuron and memory function in HT22 hippocampal cells and MCAO mice. Screening for selection of functional herbs on memory impairment was performed using HT22 cells. Among many herbal candidates, we found significant neuroprotective effects of *Cynanchum wilfordii*, which is known to play a key role in preventing vascular disorders, ischemia-induced disease, and aging progress by Joon Hur in Donguibogam [17]. Therefore, we used CWC-9 including *Cynanchum wilfordii* and the above mentioned multi-herb formulae to enhance medical efficiency.

Exposure of HT22 cells to glutamate evokes neuronal cell death via non-receptor-mediated oxidative stress [19]. High levels of ROS production caused by exposure to glutamate lead to both apoptotic and necrotic processes [23] and the subsequent oxidative stress can cause cellular damage and death in cerebral ischemia and reperfusion [9]. In particular, hippocampus, one of the brain regions most sensitive to ischemic injury, plays a key role in learning and memory [3, 4]. Our results indicate that cell death promoted by glutamate toxicity could be ameliorated by the suppression of ROS formation after treatment with CWC-9 and support a neuroprotective role and therapeutic potential for a new herbal mixture extract. In addition, the Morris water maze and passive avoidance task were performed to assess

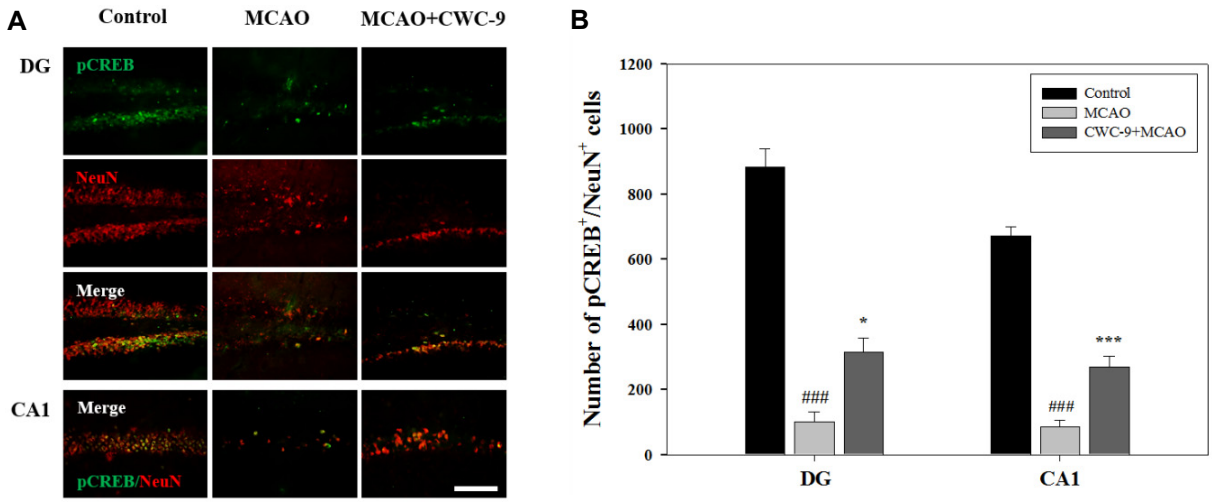

Fig. 6. Effect of CWC-9 on pCREB protein expression in the hippocampus at 26 days after MCAO. Photomicrograph (A) and its histogram for pCREB/NeuN (B) double-positive cells in the DG and CA1 regions of the hippocampus. Total number of $pCREB^{+}/NeuN^{+}$ cells was significantly increased by administration of CWC-9 in DG and CA1 compared to the MCAO group. ### $p<0.001$ vs. control, * $p<0.05$ and *** $p<0.001$ vs. MCAO mice. Scale bar = 100 μm.

MCAO-induced deficits of memory. Treatment of MCAO mice with CWC-9 resulted in a significant reduction in time and distance from 22 to 25 days compared with the MCAO group by the water maze test. In the passive avoidance test, the step-through latency time was prolonged by treatment with CWC-9 compared with the MCAO group. These results suggest that CWC-9 may be a good candidate for recovery of damaged memory and cognitive function in a focal cerebral ischemia model.

MAPK and PI3K signaling pathways in neuronal cell death or neurodegenerative diseases play an important role in neuroprotective effects against oxidative stress [1]. In particular, roles of cell death (p38 MAPK) [15] and survival pathways (PI3K) [8, 21] are essential for cellular defense under oxidative stress conditions. Our results showed that neuroprotective effects of CWC-9 are regulated by signaling pathways of p38 MAPK and PI3K after cerebral ischemic injury.

Critical transcription factor CREB modulates adaptive neuronal responses [2] and impaired CREB phosphorylation in hippocampus may be a pathological component in neurodegenerative disorders [7, 27]. In our examination of the hippocampus using Western blot analysis, protein levels of CREB phosphorylation were recovered in CWC-9-treated MCAO mice compared with vehicle. Immunohistochemical detection of the activation of CREB resulted in significant expression in the hippocampal CA1 and DG regions after CWC-9 treatment, suggesting that enhanced CREB phosphorylation by CWC-9 treatment plays a central role in survival of hippocampal neuronal cells.

At present, the main functional components of multi-herb formula CWC-9 are not known. However, a methanolic extract from dried roots of *Cynanchum wilfordii* significantly mitigates the neurotoxicity induced by exposure to glutamate that participates in the cellular defense against oxidative stress [17]. Neuroprotective compound, cynandione A, from *Cynanchum* species (*C. wilfordii* and *C. auriculatum*) can attenuate ischemic brain injury and promote functional recovery [11, 28]. Moreover, the multi-herb formulae, including the roots of *Rehmannia glutinosa*, *Polygala tenuifolia,* and *Acorus gramineus* can improve the mental or physical symptoms in the elderly [20]. CWC-9 may have synergistic memory function to target primary neuroprotective effects by diverse roles within multi-herb formulae. Our results demonstrate that CWC-9 exerts significant beneficial effects on glutamate-induced HT22 cell death and ischemia-reperfusion injury model.

In conclusion, this study demonstrates that CWC-9, a new herbal extract mixture, protects hippocampal neuronal cells via suppression of ROS generation with regulation of the signaling pathways of p38 MAPK and PI3K associated with CREB phosphorylation. These effects can also improve memory and cognitive function against cerebral ischemic stroke. These results have shown that multi-herb formula CWC-9 may be a useful therapeutic agent in treatment of various brain disorders associated with changes in memory and cognition.

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초록 : 한약재 4종 복합추출물의 해마신경세포 보호를 통한 기억력 개선

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본 연구는 동의보감을 근거로 선별된 백수오, 지황, 원지 및 석창포로 구성된 복합추출물의 해마신경세포에 대한 보호 및 기억력의 개선효과를 살펴보았다. 복합추출물의 신경보호효과를 검증하기 위해 HT22해마신경세포의 생존율, 세포독성 및 활성산소를 분석하였으며, 허혈성 뇌손상 마우스모델을 이용하여 기억에 대한 동물행동학적 변화와 단백질 발현을 측정하였다. 해마신경세포에서 복합추출물의 전 처리는 glutamate에 의해 유도된 활성산소의 축적을 억제하였으며 세포사멸을 감소시켰다. 허혈성 뇌손상 마우스모델에서 복합추출물은 동물행동학적으로 공간 및 단기 기억능력을 개선시켰다. 뇌허혈로 인해 증가된 p38 MAPK의 인산화는 복합추출물에 의해 현저히 감소하는 반면, 감소된 PI3K와 CREB의 인산화는 현저히 증가하였다. 이를 면역조직화학분석을 통해 복합추출물을 투여한 그룹이 해마에서 발현되는 CREB의 인산화가 현저히 증가되는 것을 확인하였다. 이상의 결과는 복합추출물이 CREB 단백질과 관련된 신경보호 신호기전을 조절함으로써 인지기능을 개선시키는 것으로 사료된다.