

Full Paper

Chotosan, a Kampo Formula, Ameliorates Chronic Cerebral Hypoperfusion-Induced Deficits in Object Recognition Behaviors and Central Cholinergic Systems in Mice

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Abstract. We previously demonstrated that the Kampo formula chotosan (CTS) ameliorated spatial cognitive impairment via central cholinergic systems in a chronic cerebral hypoperfusion (P2VO) mouse model. In this study, the object discrimination tasks were used to determine if the ameliorative effects of CTS on P2VO-induced cognitive deficits are a characteristic pharmacological profile of this formula, with the aim of clarifying the mechanisms by which CTS enhances central cholinergic function in P2VO mice. The cholinesterase inhibitor tacrine (THA) and Kampo formula saikokeishito (SKT) were used as controls. P2VO impaired object discrimination performance in the object recognition, location, and context tests. Daily administration of CTS (750 mg/kg, p.o.) and THA (2.5 mg/kg, i.p.) improved the object discrimination deficits, whereas SKT (750 mg/kg, p.o.) did not. In ex vivo assays, tacrine but not CTS or SKT inhibited cortical cholinesterase activity. P2VO reduced the mRNA expression of m3 and m5 muscarinic receptors and choline acetyltransferase but not that of other muscarinic receptor subtypes in the cerebral cortex. Daily administration of CTS and THA but not SKT reversed these expression changes. These results suggest that CTS and THA improve P2VO-induced cognitive impairment by normalizing the deficit of central cholinergic systems and that the beneficial effect on P2VO-induced cognitive deficits is a distinctive pharmacological characteristic of CTS.

Keywords: chotosan, chronic cerebral hypoperfusion, object recognition memory deficit, cholinergic system, mouse model

Introduction

Vascular dementia accounts for about a quarter of people with dementia in Japan (1). A large number of psychotropic drugs have been used to ameliorate the neuropsychiatric symptoms that accompany vascular dementia. However, very few drugs have been found that can cure this disorder.

Chotosan (CTS) is a Kampo prescription consisting of 10 different medicinal herbs and gypsum fibrosum. This prescription has been long used for the treatment of chronic headache and hypertension, particularly in middle-aged or older patients who have weak physical constitutions, chronic headache, painful tension of the shoulder and cervical muscles, vertigo, morning headache, a heavy feeling of the head, flushing, tinnitus, and insomnia (2). In a recent clinical study based on a double blind and placebo-controlled paradigm, it was demonstrated that CTS has an ameliorative effect on cognitive dysfunctions in stroke patients (2, 3). Moreover, pharmacological studies have reported that CTS prevents the occurrence of stroke, prolongs the life span of stroke-prone spontaneously hypertensive rats (4, 5), and reduces tissue damage associated with ischemia reperfusion. Furthermore, polyphenolic compounds and some alkaloid components have been reported to play an

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important role in cytoprotection against tissue damage mediated by oxidative stress (6) and excessive excitatory synaptic transmission (7, 8). However, very little information is available about the mechanisms underlying the therapeutic effects of CTS in stroke patients.

Two vessel occlusions of common carotid arteries (P2VO) induce progressive spatial memory deficits and neuronal damage in rodents (9 – 12), and this model can be used to assess the nootropic action of drugs (13 – 15). In our previous study (16) using a mouse model of P2VO, we found impairments of spatial learning and memory and a reduced level of acetylcholine (ACh) in the brain. Moreover, we found that daily administration of CTS and tacrine (THA) attenuated learning and memory deficits in the P2VO mouse brain by elevating ACh levels and thereby indirectly stimulating muscarinic M1 receptors. These findings prompted us to investigate i) if the beneficial effect of CTS observed in P2VO mice is a distinctive pharmacological profile of this prescription, ii) whether CTS improves not only spatial but also non-spatial cognitive deficits in a mouse model of vascular dementia, and iii) how CTS administration enhances central cholinergic functions impaired by P2VO. In this study, we employed an object recognition test as a behavioral paradigm to elucidate object recognition, object location, and contextual memory in mice with P2VO-induced cerebral hypoperfusion and to examine the effects of CTS on these types of memory deficits. The cholinesterase inhibitor THA was used as a control because previous studies have demonstrated that THA administration improves learning and memory deficits elicited in an animal model of chronic cerebral hypoperfusion (15 – 17). We also compared the effects of CTS with those of the Kampo prescription saikokeishito (SKT) since the clinical use of SKT is different from that of CTS. Indeed, SKT is used for the treatment of diseases such as influenza virus infection, pneumonia, and gastric ulcer (18) but not for the treatment of chronic headache and hypertension.

Materials and Methods

Drugs

CTS and SKT used in this study were obtained from Tsumura & Co. (Tokyo) in the form of a spray-dried powder extract, which were each prepared using the standardized extraction method of medicinal plants registered in the Japanese Pharmacopoeia XV.

CTS was extracted from a mixture of 3.0 parts Uncariae Uncis cum Ramulus (hooks and branch of Uncaria rhynchophylla MIQUEL), 3.0 parts Aurantii Nobilis pericarpium (peel of Citrus unshiu MARKOVICH), 3.0 parts Pinelliae tuber (tuber of Pinellia ternate BREITENBACH), 3.0 parts Ophiopogonis tuber (root of Ophiopogon japonicus KER-GAWLER), 3.0 parts Hoelen (sclerotium of Poria cocos WOLF), 2.0 parts Ginseng radix (root of Panax ginseng C. A. MEYER), 2.0 parts Saphoshnikoviae radix (root and rhizome of Saposhnikovia divaricata SCHISCHKIN), 2.0 parts Chrysantheni flos (flower of Chrysanthemum morifolium RAMATULLE), 1.0 part Glycyrrhizae radix (root of Glycyrrhiza uralensis FISHER), 1.0 part Zingiberis rhizome (rhizome of Zingiber officinale ROSCOE), and 5.0 parts Gypsum fibrosum (CaSO₄ 2H₂O). SKT was extracted from a mixture of 5.0 parts Bupuleuri radix (root of Bupleurum falcatum LINNE), 4.0 parts Pinelliae tuber, 2.0 parts Scutellariae radix (root of Scutellaria baicalensis GEORG), 2.0 parts Glycyrrhizae radix, 2.0 parts Cinnamomi cortex (bark of Cinnamomum cassia BLUME), 2.0 parts Paenoniae radix (root of Paenonia lactiflora PALLAS), 2.0 parts Zizyphi fructus (fruit of Zizyphus jujuba MILLER var. inermis REHDER), 2.0 parts Ginseng radix, and 1.0 part Zingiberis rhizoma. The yields of CTS and SKT extract were 16.1% and 18.2%, respectively. Voucher specimens (CTS: Lot No. 2020047010 and SKT: Lot No. 2050010010) have been deposited at our institute.

These extracts were dissolved in distilled water and administered orally. Tacrine (THA; 9-amino-1,2,3,4-tetrahydro-acridine HCl; Sigma-Aldrich Co., St. Louis, MO, USA), a control, was dissolved in physiological saline and injected intraperitoneally. Drug administration was performed daily at around 9 a.m. during the experimental period. The doses of the drugs used in this study were selected based on our previous reports (5, 16, 19).

Animals

Male ICR mice (Japan SLC, Inc., Hamamatsu) were housed in a laboratory animal room maintained at 25 ± 1°C with 65 ± 5% humidity on a 12-h light/dark cycle (lights on: 07:30 to 19:30) for at least one week before the start of the experiments. Animals were given food and water ad libitum. A total of 170 mice were used for the experiments. All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan and had the approval of the Institutional Animal Use and Care Committee in University of Toyama.

Surgical operation

Surgical procedures were performed as previously described (16). Briefly, at the age of 8 weeks, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the bilateral common carotid
arteries were carefully separated from the cervical sympathetic and vagal nerves through a ventral cervical incision. The arteries were then ligated with silk thread in 2VO mice. Animals that received the same surgical procedure without carotid artery ligation served as sham-operated controls.

Behavioral test and drug administration

Behavioral experiments and drug administration were started 2 weeks after the surgical procedures. Object recognition test (ORT)-1 and -2: ORT was performed by slightly modifying the method described by De Rosa et al. (20). The apparatus consisted of a square arena (52 × 52 × 40 cm) made of poly (vinyl chloride) with gray walls and a white floor. The objects for recognition had visual patterns or visually different shapes to be discriminated. The box arena and objects were cleaned by 75% ethanol between trials to prevent a build-up of olfactory cues. Performance of the animals in this test was video-recorded for later analysis.

The animals received three sessions of 10-min duration in the empty box to acclimatize them to the apparatus and test room. The ORT-1 consisted of a sample phase trial and a test phase trial. In the sample phase trial, each mouse was first placed in the box for 5 min and exposed to two identical sample stimuli, objects O1 and O2 (each 5 × 5 × 5 cm white cubes) for 5 min. The total time the mouse spent exploring each of two objects was measured and then the mouse was returned to its cage. Test phase trials were performed 24 h after the sample phase trials. In this trial, one of the two objects was replaced by its identical copy (objects F) and the other by a new one with a black-and white pattern (object N). The total time spent exploring each of the two objects was measured.

After the ORT-1, P2VO mice were randomly separated into 4 groups and received daily administration of vehicle or test drugs for 5 days. The sham-operated group was administered vehicle daily. ORT-2 was conducted at day 6 after ORT-1. In the sample phase trial of ORT-2, an animal was placed into the arena where two identical sample objects made of glass (approx. 5 × 5 × 6 cm in size) were placed in two adjacent corners of the arena and the animal was allowed to explore for 5 min and then returned to its cage for 10 min of a retention interval. In the test phase trials, the objects were replaced with two new objects: one was identical to that used in the sample phase trial and the other was a novel object that the mice had never encountered before. Mice were left to explore the objects for 5 min and the total time spent exploring each of the two objects was measured.

Object location test (OLT): The sample phase trials of the OLT were conducted in the same way as those of the ORT. The objects used in the sample phase trials were two black cones (A1 and A2: 5-cm diameter and 10-cm height each). After a delay of 10 min, the test phase trials were conducted. In this trial, the objects were replaced by their identical copies, one of which was placed in the same position, whereas the other one was moved to the adjacent corner, so that the two objects were now in diagonally opposite corners. In the test phase trials, both objects were equally familiar to the animals, but one had changed location. The mice were exposed to the objects for 5 min and the total time spent exploring each of the two objects was measured.

Object context test (OCT): The OCT was used to determine whether mice were sensitive to a change in context for a given object. In this test, two open field arenas (50 × 50 × 40 cm) made of poly (vinyl chloride) were used. Each arena (condition A and condition B) consisted of different walls and floors. In condition A, the arena consisted of black walls with horizontal stripes and a rough black wooden floor and two identical white cubes were placed as objects (A1 and A2), while in condition B, the arena consisted of gray walls and a rough gray wooden floor and two identical black cones were placed as objects (B1 and B2). In each session, P2VO-treated and untreated mice were exposed to both conditions (A and B). The OCT consisted of four sample phase trials and one test phase trial, and each phase lasted for 5 min. The retention interval within the sample phase trials was 3 min. There was a 10-min interval between the last sample phase and the test phase trials. In the sample phase trial, two objects were placed in adjacent corners of the arena; phases 1 and 4 comprised objects A1 and A2 in environment A, and phases 2 and 3 comprised objects B1 and B2 in environment B. The test phase was performed in the same environment as the sample phase 4, but one of the objects (A2) was replaced by B2. In this way, one object was in the same environment as in the sample phase trial, and the other object was in a different environment from the sample phase trial.

The exploration of an object was defined as directing the nose to the object at a distance of <2 cm and touching it with the nose according to a previous report (20). Turning around, climbing over, or sitting on the object were not included. If the exploration time in the sample phase trials was <3 s, the mice were discarded from the sample. Moreover, if the mice spent <1 s exploring both new and familiar objects in the test phase trials, they were also excluded from the sample (20). A discrimination index (DI) was calculated according to the following equation (12, 20):

\[ DI = (T_n - T_f) / (T_f + T_n) \]
Here, $T_s$ and $T_f$ represent the time spent to explore new and familiar objects during a 5-min observation period, respectively.

**Ex vivo analysis of cholinesterase activity in the brain**

The normal mice were subjected to administration of test drugs for five consecutive days. Mice were divided into four groups and each group received only water, CTS (750 mg/kg per day, p.o.), SKT (750 mg/kg per day, p.o.), or THA (2.5 mg/kg per day, i.p.) for 5 consecutive days. On day 6, the animals were decapitated and the frontal cortices were dissected out and kept at −80°C until use.

Determination of cholinesterase activity was performed based on the colorimetric method originally described by Ellman et al. (21). Briefly, the frozen cortex was weighed and homogenized in 10-times volume of 0.1 M phosphate buffer (pH 7.4) containing 1% Triton-X-100. After centrifugation at 15,000 rpm at 4°C for 20 min, the clear supernatants were removed and served as the enzyme source. Cholinesterase activity was determined in 50 µl aliquots of the homogenates (run as duplicates) in 96-well flat-bottom microplates. The reaction was started by adding 20 µl 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 20 µl 30 mM acetylthiocholine, and 160 µl phosphate buffer. The spectrophotometric absorption at 405 nm during a 3-min incubation period at 25°C was quantitatively measured using a microplate reader (Sunrise Classic; TECAN Japan, Kawasaki) and expressed as nmol ACh hydrolyzed / (min·mg tissue).

**Reverse transcriptase-polymer chain reaction (RT-PCR) assay for cholinergic markers**

All mice were decapitated after completing the behavioral studies. The brain was removed immediately after decapitation, and the cerebral cortex and hippocampus were dissected out and kept at −80°C until use. Total RNA was extracted from the hippocampus and cortex using Sepazol® (Nacalai Tesque, Kyoto) according to the manufacturer’s instructions. First-strand cDNA synthesis was conducted using oligo (dT) primers and M-MLV Reverse Transcriptase® (Invitrogen, Rockville, MD, USA) in a total volume of 20 µl. The reaction was performed at 25°C for 10 min and heated at 37°C for 60 min and 98°C for 5 min before cooling to 4°C. β-Actin was used as a housekeeping gene control. PCR was conducted using 1 mM of primer sets, 250 mM dNTPs, and 2 units of Taq DNA polymerase (Promega, Madison, WI, USA) in reaction buffer containing 2.5 mM MgCl₂. Thermocycling was performed using the following protocol: 1) 94°C for 3 min; 2) designated cycles of 94°C for 30 s, 57°C–63°C for 1 min, and 72°C for 1 min; and then 3) 72°C for 5 min before cooling to 4°C. The following primers were synthesized by Nippon EGT Co. (Toyama): choline acetyltransferase (ChAT, NM_009891), cctgtaaacttctactgtag (sense), and gtagctaagcacacc agagatgag (antisense); m₁ isoform (M16406): actgtctggcaccaggaa (sense) and tctag gcgaatatcagag (antisense); m₂ isoform (NM_031016): tgcattctactagtcg (sense) and gaggcttgctgacacat (antisense); m₃ isoform (NM_012527): acatctctgtag tgccc (sense) and tagtgatgctgtagt (antisense); m₄ isoform (M18088): gagaagggcagactctg (sense) and aggactgagttgactgcc (antisense); m₅ isoform (M22926): ccatcttggcgactc (sense) and gttcttctgtagt (antisense); β-actin (accession No. V01217): gttctggtctgccc (sense) and aacgtctcaacgta (antisense). To allow co-amplification of target genes (ChAT gene and muscarinic receptor isoform genes) with the more abundant β-actin mRNA, pilot studies were conducted to determine optimal relative primer concentration and cycle number whereby the PCR would still be within the exponential phase (i.e., within the linear ranges) of amplification for all transcripts according the method described by Uz et al. (22). Based on the pilot studies in which we ran specific primers for both target genes and β-actin in the same PCR mixture, the proper cycle number for the PCR amplification of each target gene tested was chosen (β-actin: 22, ChAT: 32, m₁: 27, m₂: 29, m₃: 27, m₄: 28, m₅: 29 PCR cycles).

After electrophoresis, PCR products were semi-quantitated by comparing them with the signal of β-actin mRNA using the Image J program (ver. 1.34S).

**Statistical analyses**

All data were expressed as the mean ± S.E.M. Statistical analyses of the behavioral data comprised paired and unpaired Student’s t-tests and a one-way analysis of variance (ANOVA) or two-way ANOVA followed by a post-hoc multiple comparison test (the Student-Newman-Keuls test), as appropriate. The mRNA expression level and cholinesterase activity were analyzed with Student’s t-test or a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Differences of $P<0.05$ were considered significant.

**Results**

**CTS improves novelty preference impairments in the ORT**

Using a novel object recognition test, we first elucidated the effect of P2VO on learning and memory performance which is related to episodic memory in humans. Analysis of performance in the sample phase trials of the ORT revealed no differences in total explor-
ing time between sham-operated and P2VO mice [30.6 ± 3.0 s (mean ± S.E.M., n = 12) in sham-operated and 28.1 ± 1.4 s (n = 45) in P2VO; t = 0.842, df = 55, P = 0.404, t-test] (Fig. 1). These results eliminate the possibility that chronic cerebral hypoperfusion caused by P2VO treatment causes deficits of motivation and sensory motor function. However, in the test phase trials conducted 24 h after the sample phase trial, the sham-operated mice spent a significantly longer time exploring a new object than exploring a familiar object (t = −6.328, df = 9, P < 0.01, paired t-test), whereas vehicle-treated P2VO mice did not show a significant difference (t = 1.634, df = 11, P = 0.131, paired t-test). However, P2VO mice treated with CTS and THA spent a significantly longer exploring time dedicated to the novel object than the time spent exploring a familiar object (CTS: t = −2.853, df = 10, P < 0.05; THA: t = −4.953, df = 10, P < 0.01, paired t-test). On the other hand, the animals treated with SKT showed no difference in the exploratory time between novel and familiar objects (SKT: t = −0.214, df = 10, P = 0.835, paired t-test) (Fig. 2). These data demonstrated that P2VO mice fail to discriminate a novel object, whereas CTS-
THA- but not SKT-treated P2VO mice are clearly able to discriminate between the two objects. In fact, the post hoc analysis following one-way ANOVA \[F(3,41) = 14.969, P < 0.01\] revealed that the CTS-treated group had a DI significantly higher than those of the P2VO group (Fig. 2).

**CTS improves impaired performance of P2VO mice in the OLT**

Analysis of the sample phase revealed that there were no significant differences in the total amount of exploration time among the sham-operated group and P2VO groups treated with vehicle or test drugs \[F(4,49) = 0.086, P = 0.99\], one-way ANOVA] (Fig. 3). In the test phase trials, exploration times for the sham-operated group and the CTS- and THA-treated P2VO groups clearly showed a preference for an object placed in a novel location compared to an object placed in a familiar location (sham-operated: \(t = -6.660, df = 9, P < 0.01\); CTS-treated P2VO: \(t = -3.676, df = 10, P < 0.01\); THA treated-P2VO: \(t = -3.306, df = 10, P < 0.01\), paired t-test) (Fig. 3). In contrast, the vehicle- and SKT-treated P2VO groups showed that they were unable to discriminate a novel location from a familiar location or spent more time in a familiar location (vehicle-treated P2VO: \(t = 3.332, df = 10, P < 0.01\); SKT-treated P2VO group: \(t = 0.289, df = 10, P = 0.778\), paired t-test). One-way ANOVA of the DI among the P2VO groups revealed a
significant effect of drug treatment \([F(3,40) = 13.69, P < 0.01]\), and CTS and THA administration significantly reversed DI values to the levels of the sham-operated group (Fig. 3).

**CTS improves impaired performance of P2VO mice in the OCT**

In the sample phase of an object context version test (Fig. 4), we first analyzed the data using two-way ANOVA [operation vs. environment] taking into account both the environmental and operation factors. Analysis showed that there was no significant difference for either factor \([F_{\text{operation}}(1,42) = 0.12, P = 0.737; F_{\text{environment}}(1,42) = 0.051, P = 0.82]\). The effect of different operation did not depend on what environment was present. There was no statistically significant interaction between these factors in the sample phase trials \([F_{\text{operation} \times \text{environment}}(1,42) = 0.904, P = 0.347]\), indicating no significant preferences between the two environments (Fig. 4). In the test phase trials, a paired \(t\)-test was employed to verify the eventual difference in the exploration time between the object that was placed in the changed environment (target object) and the object that was in the same environment (old object) (Fig. 4). The sham-operated group (\(t = -6.154, \text{df} = 11, P < 0.01\), paired \(t\)-test) and the CTS- (\(t = -3.418, \text{df} = 11, P < 0.01\), paired \(t\)-test) and THA-treated P2VO groups (\(t = -3.258, \text{df} = 10, P < 0.01\), paired \(t\)-test) were capable of discriminating between the old and target objects, while no significant differences were found in the vehicle- (\(t = 1.769, \text{df} = 10, P = 0.107\), paired \(t\)-test) and SKT-treated P2VO groups (\(t = 0.683, \text{df} = 10, P = 0.510\), paired \(t\)-test). One-way ANOVA of the DI among the P2VO groups revealed a significant effect of drug
treatment in the P2VO groups \[F(3,40) = 6.317, P < 0.01\], and CTS and THA administration significantly reversed DI values to the level of the sham-operated group (Fig. 4).

**Effects of CTS on cholinesterase activity in the cortex**

Considering our previous findings that CTS- and THA-treated P2VO mice had an elevated level of ACh content in the brain, we examined the ex vivo effects of CTS on acetylcholinesterase activity in the brain. One-way ANOVA revealed an overall group difference in cholinesterase activity \[F(3,20) = 6.84, P < 0.01\] (Table 1). Post hoc analysis showed that daily administration of THA (2.5 mg/kg, i.p.) for 6 days significantly reduced cholinesterase activity, while CTS and SKT treatment did not have any significant effect on the activity.

**Table 1.** Effect of repeated administration of chotosan (CTS), saikokeishito (SKT), and tacrine (THA) on cholinesterase activity in the cortex

<table>
<thead>
<tr>
<th>Treatment (dose)</th>
<th>Cholinesterase activity [nmol / (mg tissue ⋅ min)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.57 ± 1.52</td>
</tr>
<tr>
<td>CTS (750 mg/kg per day, p.o.)</td>
<td>18.22 ± 1.39</td>
</tr>
<tr>
<td>SKT (750 mg/kg per day, p.o.)</td>
<td>23.25 ± 1.88</td>
</tr>
<tr>
<td>THA (2.5 mg/kg per day, i.p.)</td>
<td>13.28 ± 2.15**</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. \((n = 6)\). **\(P < 0.01\), compared with the control.

**Effects of CTS on expressions of cholinergic marker genes**

**CHAT mRNA expression:** To clarify the mechanism(s) underlying the ameliorative effects of CTS on P2VO-
Effects of CTS, SKT, and THA on P2VO-induced changes in choline acetyltransferase (ChAT) mRNA expression in the cerebral cortex. After completing the behavioral studies, the animals were decapitated and total RNAs were prepared from the cerebral cortices. Synthesis of the first strand DNA from total RNA samples and PCR were conducted as described in the text. A: Representative changes in ChAT mRNA expressions. Each PCR product was electrophoresed in duplicate on polyacrylamide gels. M: DNA size marker. B: Semiquantitative analysis of ChAT mRNA expression in sham and P2VO mice treated with vehicle, CTS (750 mg/kg per day), SKT (750 mg/kg per day), and THA (2.5 mg/kg per day). Each data column represents the mean ± S.E.M. obtained from 5 – 6 brain samples each. **P<0.01 vs vehicle-treated sham-operated group. ***P<0.01 vs vehicle-treated P2VO group.

Discussion

This study has demonstrated that chronic cerebral hypoperfusion induced by P2VO produces non-spatial and spatial cognitive deficits in an object recognition task and down-regulates the expression of cholinergic marker genes encoding ChAT and muscarinic m1 and m5 receptors in the brain. Furthermore, daily administration of CTS post operation improves cognitive deficits and normalizes expression levels without inhibiting cholinesterase activity. The present findings suggest that CTS improves chronic cerebral hypoperfusion-induced cognitive deficits in part by normalizing the impaired function of cholinergic systems in the brain and raise the possibility that the beneficial effect of CTS on cognitive deficits may be a result of the distinctive pharmacological profile of this Kampo medicine.

In this study, we employed an object recognition task to investigate the effect of P2VO-induced cerebral hypoperfusion on visual recognition memory in mice because paradigms used in this task are available not only to analyze performance to explore a novel object but also to measure other aspects such as recognition of object location and context. In the sample phase trial of the object recognition task, no significant difference in total exploring time spent for identical objects was observed between sham-operated and P2VO mice. In addition, there were no differences in preferences between each identical object in the sham-
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operated and P2VO mice, indicating no virtual differences in vision and/or an ability to visually recognize objects between these animal groups. In contrast in the test phase trials, it was clearly revealed that sham-operated mice spent more time exploring a novel object, whereas P2VO mice failed to show a novelty preference, indicating that P2VO causes an object recognition memory deficit in mice.

Fig. 6. Effects of CTS, SKT, and THA on P2VO-induced changes in muscarinic m₁-, m₃-, and m₅-receptor mRNA expressions in the cerebral cortex. Left panels: Representative changes in muscarinic m₁- (1), m₃- (2), and m₅-receptor (3) mRNA expressions. Each PCR product was electrophoresed in duplicate on polyacrylamide gels. M: DNA size marker. Right panels: Semiquantitative analysis of muscarinic m₁-, m₃-, and m₅-receptor mRNA expression in sham and P2VO mice treated with vehicle, CTS (750 mg/kg per day), SKT (750 mg/kg per day), and THA (2.5 mg/kg per day). Each data column represents the mean ± S.E.M. obtained from 5 – 6 brain samples each. **P<0.01 vs vehicle-treated sham-operated group. *P<0.05 and ***P<0.01 vs vehicle-treated P2VO.

Table 2. Effects of chotosan (CTS), saikokeishito (SKT), and tacrine (THA) on P2VO-induced changes in expression level of muscarinic receptor subtype mRNAs in the hippocampus

<table>
<thead>
<tr>
<th>Subtype gene</th>
<th>Sham water</th>
<th>CTS</th>
<th>SKT</th>
<th>THA</th>
</tr>
</thead>
<tbody>
<tr>
<td>m₁</td>
<td>0.96 ± 0.14</td>
<td>0.75 ± 0.06</td>
<td>0.86 ± 0.06</td>
<td>0.74 ± 0.09</td>
</tr>
<tr>
<td>m₂</td>
<td>0.99 ± 0.32</td>
<td>0.87 ± 0.12</td>
<td>0.78 ± 0.17</td>
<td>0.87 ± 0.14</td>
</tr>
<tr>
<td>m₃</td>
<td>1.22 ± 0.18</td>
<td>0.47 ± 0.06**</td>
<td>1.04 ± 0.19*</td>
<td>0.90 ± 0.10</td>
</tr>
<tr>
<td>m₄</td>
<td>1.13 ± 0.04</td>
<td>0.88 ± 0.06</td>
<td>0.93 ± 0.13</td>
<td>1.04 ± 0.16</td>
</tr>
<tr>
<td>m₅</td>
<td>0.94 ± 0.16</td>
<td>0.42 ± 0.09**</td>
<td>0.70 ± 0.09**</td>
<td>0.74 ± 0.14</td>
</tr>
</tbody>
</table>

Each value represents the expression ratio (the mean ± S.E.M., n = 5 – 6) of each muscarinic receptor subtype mRNA compared to β-actin mRNA. **P<0.01, compared with the sham-operated group. *P<0.05, **P<0.01, compared with the vehicle-treated P2VO group.
One of the important findings in this study is that when administered daily to the P2VO animals with object recognition memory deficits, CTS and THA could improve the ability to discriminate between familiar and new objects in the second object recognition test. These findings suggest that CTS has a beneficial effect on recognition memory deficits caused by chronic cerebral hypoperfusion in mice. This view is supported by the data obtained in the object location and contextual tests. Lines of evidence indicate that normal rodents spend more time exploring an object placed in a novel location than in a familiar location in an object location test. Moreover, their performance in the test trial is relevant to spatial cognitive memory attributable to the hippocampus system involved in detecting spatial novelty (20, 23, 24). In the test phase trial, sham-operated control animals showed a higher preference for an object placed in a novel location than for an object placed in a familiar location, whereas the vehicle-treated P2VO mice were unable to discriminate the object placed in a novel location. Thus, together with the previous reports that P2VO animals exhibit learning and memory deficits in the water maze and radial maze tasks (9, 16, 17), these results suggest that the impaired performance of P2VO mice in an object location test is due to spatial cognitive deficits induced by chronic cerebral hypoperfusion. In addition, vehicle-treated P2VO mice were found to be insensitive to the combination of objects and environment. Indeed, they were unable to link exploration of an object with an environment that served as a contextual cue. Mumby et al. (25) have elucidated the memory of rats for objects, places, and context using three types of paradigms of object recognition tests and demonstrated that hippocampal damage impairs memory for contextual or spatial aspects of an experience, whereas object recognition memory is left intact. Moreover, there are reports indicating an important role of cholinergic input to the cortex in object recognition behavior in rodents (26 – 28). Therefore, it is likely that impairment of object recognition performance in P2VO mice is attributable not only to a dysfunction of the hippocampal system but also to deficits in the brain regions other than the hippocampus.

Interestingly, it was found that daily administrations of CTS and THA to P2VO mice are able to ameliorate object recognition memory deficits elicited by P2VO in
both object location and object contextual test. Lines of evidence have indicated that dysfunction of central cholinergic systems impairs object recognition memory in rodents. Drugs such as cholinesterase inhibitors, which facilitate cholinergic function, improve the memory deficits observed in object recognition tests, suggesting that the integrity of the cortical cholinergic system is required for high object recognition performance (26, 29, 30). We previously reported that CTS improved P2VO-induced spatial learning deficits in part by facilitating the cholinergic system and that CTS as well as THA reversed P2VO-induced decreases in ACh content in the brain (16). Therefore, one may infer that CTS, like THA, ameliorates not only spatial cognitive deficits but also non-spatial recognition memory impairment in P2VO mice via activation of cholinergic systems in the brain.

In this study, we used SKT as a reference Kampo prescription because although this prescription includes 4 kinds of medicinal plants in common with CTS, it has been clinically used to treat cold, pneumonia, gastric ulcer (18), and inflammatory diseases (31), but CTS is not prescribed because it does not target these disorders. In contrast to the effects of CTS and THA, daily administration of SKT failed to affect recognition memory deficits induced by P2VO in the three types of paradigms of the object recognition test. Moreover, in our preliminary study using a water maze task, daily SKT administration to P2VO mice during a training period had no effect on spatial cognitive deficits caused by P2VO (data not shown). Therefore, considering differences in the pharmacological effects between CTS and SKT, the amelioration of spatial and non-spatial cognitive deficits may be a result of the distinctive pharmacological profile of CTS.

To better understand the mechanisms underlying the action of CTS, we investigated the effect of CTS on ex vivo cholinesterase activity in the mouse cortex based on both the basis of our previous (16) and present data, which suggest the involvement of the central cholinergic system in the action of CTS. Repeated administration of THA (2.5 mg/kg, i.p., once daily for 5 days) to normal mice significantly inhibited cholinesterase activity in the cortex, whereas neither CTS nor SKT had a significant effect on this activity. These findings raise the possibility that facilitation of central cholinergic systems induced by CTS involves different mechanism(s) from those induced by THA and lead us to infer that cholinesterase inhibition is not always the mechanism responsible for the beneficial effect of CTS on P2VO-induced cognitive deficits in mice. However, further investigations using hippocampal tissues as an enzyme source may be required to more thoroughly answer questions concerning involvement of cholinesterase inhibition in the effects of CTS since cholinergic hypofunction impairs spatial cognitive behavior in which the hippocampus plays an important role (24, 25, 32).

Importantly, the ChAT mRNA expression in the cortex was reduced by P2VO and daily administration of CTS and THA reversed the P2VO-induced down-regulation of ChAT mRNA expression in this study. These findings seem to support the previous findings concerning CTS- and THA-induced reversal of brain ACh content decreased by P2VO (16) and suggest that CTS and THA normalize brain ACh content in P2VO mice in part by increasing ChAT mRNA expression and thereby facilitate the central cholinergic systems. This mechanism may at least partly mediate the ameliorative effects of CTS and THA on spatial and non-spatial cognitive deficits in P2VO mice. Lines of evidence indicate that nerve growth factor (NGF), a neurotrophin, enhances the cholinergic phenotype observed as ChAT activity, immunoreactivity, and mRNA and that the transcription factor c-fos plays an important role in the NGF-mediated changes in the cholinergic phenotype (33, 34). Moreover, Kato et al. (35) have reported that ChAT activity in cultured cholinergic neurons can be upregulated by stimulation of σ receptors. Therefore, such factors are likely involved in the ameliorative effects of CTS and THA. This possibility is currently under investigation in this laboratory.

The present study revealed that chronic cerebral hypoperfusion induced by P2VO caused a significant reduction of muscarinic m1- and m5-receptor mRNA expression in the cortex and hippocampus without affecting the expression of genes encoding other muscarinic receptor subtypes (m2, m3, and m4). The reason for the different susceptibilities of these gene expressions to chronic cerebral hypoperfusion remains unclear but the present findings are partly consistent with previous data that ischemic insults caused dynamic alterations of mRNA expression of some muscarinic receptor subtypes (m1, m2, and m4) but not m5 receptors in a temporal manner (36). It is of interest to note that the P2VO-induced down-regulation of the m1- and m5-receptor mRNA expression was reversed by repeated administration of CTS and THA. The exact mechanisms by which CTS and THA normalize the expression levels of m1- and m5-receptor subtype genes are unclear. However, in vivo studies have demonstrated that the stimulation of muscarinic ACh receptors provides neuroprotection against transient ischemia-induced neuronal cell death, even when reperfusion has been carried out in an animal model of transient forebrain ischemia (37, 38). Moreover, stimulation of the m5-receptor subtype reportedly triggers protection of
ischemia-induced myocardial injuries (39) and apoptotic cerebellar granule neurons (40) in a manner reversible by m5-receptor antagonists. A recent study (41) confirmed the anti-apoptotic ability of the m5-muscarinic receptor and indicated that the poly-basic region in the short C-terminal tail conveys the cytoprotection property of the m5-muscarinic receptor. Taken together, the present results allow us to infer that an increase in endogenous ACh level by CTS and THA may enhance the activity of m3-receptor subtypes that most probably are located on neuronal cells and thereby protect m3-muscarinic receptors expressing neuronal cells from chronic cerebral hypoperfusion-induced damage.

It has been demonstrated that the m3-receptor mRNA is located in cerebral blood vessels (42). Moreover, studies using m3-receptor–gene knockout mice have suggested that the vasorelaxing effects of ACh on cerebral arteries and arterioles are mediated by endothelial m3 receptors and that activation of cholinergic vasodilator fibers can reduce neuronal damage during certain forms of cerebral ischemia (43). Therefore, it is likely that normalization of m3-receptor mRNA expression by CTS and THA may contribute to the amelioration of cognitive deficits caused by P2VO-induced chronic cerebral hypoperfusion in mice.

It should be noted that in contrast to the effects of CTS and THA, SKT had no effect on P2VO-induced changes in the gene expression of muscarinic cholinergic markers tested in this study. Considered together with the failure of SKT to ameliorate P2VO-induced object recognition deficits and the down-regulation of ChAT and m3- and m5-receptor mRNA expressions, the present results suggest that the ameliorative effects of CTS and THA on P2VO-induced cognitive deficits are attributable at least in part to the CTS- and THA-induced reversal of gene expression in these cholinergic markers that are down-regulated by P2VO. Nevertheless, further investigations are required to clarify the molecular mechanisms underlying the effects of CTS and THA on ChAT and m3- and m5-receptor mRNA expressions.

The previous report from this laboratory (16) suggested that the effect of CTS on P2VO-induced spatial cognitive deficits is mainly attributable to Uncaria, a major constituent of CTS. Together, the present findings raise a possibility that chemical constituent(s) included in this plant play a key role in the effects of CTS on object recognition behavior and expression of some cholinergic marker genes. It will be, therefore, of interest to examine whether the extract and chemical components from Uncaria and other Kampo formula containing Uncaria exhibit pharmacological effects similar to those of CTS. Such possibilities are currently under investigation in this laboratory.

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