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# Choto-san, a Kampo formula, improves chronic cerebral hypoperfusion-induced spatial learning deficit via stimulation of muscarinic $M_1$ receptor

Yukihisa Murakami[1], Qi Zhao[1], Kousuke Harada, Michihisa Tohda, Hiroshi Watanabe, Kinzo Matsumoto*

*Division of Medicinal Pharmacology, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Japan*

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**Abstract**

A recent double-blind and placebo-controlled study demonstrated a beneficial effect of Choto-san, a Kampo (traditional medicine of Japan) formula, on cognitive impairment in patients with vascular dementia. However, the neuronal mechanism underlying the therapeutic effects of this formula remains to be clarified. Using a chronic cerebral hypoperfusion model, we investigated the effect of Choto-san on cognitive dysfunction in mice to clarify its mechanism of actions. Chronic cerebral hypoperfusion was induced by permanent occlusion of both the common carotid arteries (2VO). Choto-san and *Uncaria*, a major constituent of Choto-san, caused an improvement in 2VO-induced learning deficits, whereas *Uncaria*-free Choto-san did not. The effects of Choto-san and *Uncaria* were blocked by pirenzepine, a selective muscarinic $M_1$ antagonist. In a tube-dominance test, 2VO induced increased rates of assertive behavior in mice. 2VO mice administered Choto-san showed significantly reduced rates of assertive behavior compared to vehicle-treated controls, whereas *Uncaria*-free Choto-san and *Uncaria* had little effect on 2VO-induced assertive behavior. 2VO caused a significant decrease in the level of acetylcholine (ACh) contents in the brain, and the daily administration of Choto-san or *Uncaria* raised the ACh level to that in the sham-operated controls. These results suggest that Choto-san has an ameliorating effect on the spatial memory deficit caused by chronic hypoperfusion, and that the effect is mainly attributable to *Uncaria*. Moreover, it was suggested that the effects of Choto-san and *Uncaria* are at least partly mediated by stimulation of the muscarinic $M_1$ receptor.


*Keywords:* Choto-san; Chronic cerebral hypoperfusion; Spatial memory; Muscarinic $M_1$ receptor; Mouse

## 1. Introduction

Vascular dementia accounts for about a quarter of all cases of dementia in Japan (Meguro et al., 2002). A number of psychotropic drugs have been used to ameliorate the neuropsychiatric symptoms which accompany vascular dementia such as pathologic laughing and crying, sleep disorders, and agitation. However, very few drugs are available for the actual treatment of vascular dementia.

Choto-san is a Kampo (traditional medicine of Japan) prescription consisting of 10 medicinal herbs and *Gypsum fibrosum,* used to treat chronic headache and hypertension. Choto-san is usually prescribed to middle-age patients of considerable build with a weak physical constitution, as well as for chronic headache, painful tension of the shoulders and cervical muscle, vertigo, morning headaches, a heavy feeling of the head, feelings of uprising heat, tinnitus, and insomnia. In a recent double blind-controlled study, Choto-san was demonstrated to have an ameliorating effect on cognitive dysfunction in stroke patients (Shimada et al., 1994; Terasawa et al., 1997). Pharmacological studies have revealed that Choto-san prevents the occurrence of stroke and prolongs the life span of stroke-prone spontaneously

* Corresponding author. Tel.: +81 76 434 7610; fax: +81 76 434 5056.
*E-mail address:* mkinzo@ms.toyama-mpu.ac.jp (K. Matsumoto).
[1] These authors contributed equally to this work.

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hypertensive rats (Shimada et al., 2003) and that phenolic compounds in the prescription appear to act as an antioxidant and cytoprotective agent against oxidative damage (Mahakunakorn et al., 2004). Moreover, our previous findings have suggested that the alkaloids in Choto-san protect against an ischemia-induced abolishment of spike amplitude in the hippocampal CA1 population by preventing an excessive response by *N*-methyl-D-aspartate, muscarinic $M_1$, and 5-$HT_2$ receptors in vitro (Kang et al., 2002; Kang et al., 2004). These findings could explain the mechanism by which Choto-san reduced the incidence of stroke or tissue damage associated with ischemia-reperfusion. However, very little information is available on the mechanism underlying the therapeutic effect of Choto-san on stroke patients.

Two-vessel occlusion of the common carotid arteries (2VO) induces spatial memory deficits and neuronal damage in rodents (Ni et al., 1994, 1995a; Pappas et al., 1996; Sarti et al., 2002), and this model can be used for assessing the nootropic action of drugs (Ni et al., 1995b; Nanri et al., 1998b; Murakami et al., 2000). However, there is no report on cognitive impairment in mice with 2VO. In this study, we investigated whether mice with 2VO show learning impairments, and if so, whether the impairment can be attenuated by administering Choto-san. Here we demonstrate that 2VO mice show impaired spatial learning in the water maze task and assertive behavior in the tube dominant test and that daily administration of Choto-san during a training period significantly improved the former and attenuated the latter. Moreover, we also elucidate the mechanism underlying the beneficial effect of Choto-san and discuss the role of *Uncaria* in the actions of Choto-san.

## 2. Materials and methods

### 2.1. Drugs

One day's dosage (28 g) of Choto-san for a human adult is composed of the following materials: *Gypsum fibrosum* (5 g), *Aurantii Nobilis pericarpium* (3 g), *Ophiopogonis tuber* (3 g), *Pinelliae tuber* (3 g), *Hoelen* (3 g), *Uncariae Uncis cum Ramulus* (3 g), *Ginseng radix* (2 g), *Ledebouriellae radix* (2 g), *Chrysanthemi flos* (2 g), *Glycyrrhizae radix* (1 g) and *Zingiberis rhizoma* (1 g). Voucher specimens of each sample used in this study are deposited in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University. To prepare the Choto-san extract, *Gypsum* and all the herbs except *Uncaria*, were mixed with a 10-fold volume of distilled water and left for 1 h at room temperature. The mixture was decocted for 1 h, with *Uncaria* added for the last 15 min. An extract prepared without adding *Uncaria* was used as *Uncaria*-free Choto-san. To prepare the *Uncaria* extract, *Uncariae Uncis cum Ramulus* was mixed with 10-fold its volume of distilled water, and decocted for 15 min. After filtration, each extract was freeze-dried. The yields of the extract of Choto-san, *Uncaria*-free Choto-san and *Uncaria* were 28%, 22.8% and 12.6% in terms of the dried medicinal herbs, respectively. The *Gypsum* and herbs were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). The Choto-san extract was dissolved in distilled water and orally administered to mice 60 min before the behavioral experiment. Tacrine (9-amino-1,2,3,4-tetrahydroacridine HCl; Sigma-Aldrich Co., St. Louis, MO, U.S.A.), a reference drug, was dissolved in physiological saline and injected intraperitoneally 30 min before the behavioral experiment.

### 2.2. Animals

Male ICR mice (Japan SLC Inc., Shizuoka, Japan) were housed in the 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 1 week before the start of the experiments. Animals were given food and water ad libitum. A total of 190 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 of Toyama Medical and Pharmaceutical University.

### 2.3. Surgical operation

The animals, aged 8 weeks, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and both 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 operation but without ligation of the carotid arteries served as sham-operated controls.

### 2.4. Water maze task

Mice were tested in a 1.1 m diameter circular pool. Mice were trained four trials per day on an acquisition task. Each trial consisted of placing the mouse in the pool at one of four start positions 90° apart around the edge of the pool and allowing the mouse to swim to the hidden transparent platform (7 cm in diameter). If the mouse had not found the platform after 60 s, it was placed onto the platform by the experimenter. The mouse was allowed to remain on the platform for 10 s before being removed to an opaque high-sided plastic chamber for 60 s. The next trial was then performed. For each trial, the latency to reach the platform, distance covered, and mean swim speed were recorded via video capture and image analysis using the SMART® system (Panlab, S.L., Barcelona, Spain). The data for each day were averaged over the four trials before being used for statistical analysis. One day after the acquisition trials, a single 60 s probe trial was run in which the platform was removed from the pool. The amount of time spent in each of

the four imaginary quadrants of the pool was recorded. After the probe trial, a visible platform trial was performed with the platform placed on the side of the pool opposite its location during hidden platform training to check the vision of all mice.

### 2.5. Tube-dominance test

The tube-dominance test employed a clear plastic tube (45 cm long and 3.6 cm in diameter) and a guillotine door inserted at the center of the tube, as previously described (Lindzey et al., 1961; Lijam et al., 1997; Rodriguiz et al., 2004). The tube was trisected by imaginary transverse lines X and Y. A test mouse and a weight-matched naïve mouse were placed at the center toward the guillotine door. When the guillotine door is opened, both mice begin to explore in a forward direction. If one mouse is dominant and the other subordinate, the dominant animal will push the subordinate one back. If the test mouse pushes the naïve mouse back to the transverse line X or Y during a 1-min observation period, the event is recorded as dominance. Each dyad was tested four times (4 trials). Percent 'dominance' was then calculated.

### 2.6. Assay of acetylcholine (ACh) content of the brain

After completion of behavioral experiments, mice were sacrificed by a focal irradiation of microwaves with a strength of 7 kW for 0.9 s using a microwave applicator (Model TMW-4012A, Toshiba, Tokyo, Japan) as previously described (Xu et al., 2000). After decapitation, the brain was removed and dissected into 2 regions: the cerebral cortex and hippocampus. These tissues were quickly frozen in liquid nitrogen, weighed and homogenized in 1 mL of an ice-cold 0.1 N perchloric acid solution containing 2 mM ethylhomocholine (as an internal standard) and 0.1 mM $Na_2EDTA$ with a Polytron homogenizer (PT-10, Kinematica, Switzerland). The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. Following centrifugation, the supernatant was extracted with diethylether and then centrifuged at 2000 × *g* for 2 min. The water phase was filtered through a 0.45 μm filter (Cosmospin filter H, Nacalai Tesque Inc., Kyoto, Japan). The amount of ACh in each sample was determined using HPLC-ECD in conjunction with an enzyme reactor (Eicom, Kyoto, Japan).

### 2.7. Statistical analysis

All results are expressed as the mean ± S.E.M. The data were analyzed using a two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons among different groups for the acquisition phase of the water maze task. The data obtained from the tube-dominance test were analyzed with the $\chi^2$ test. The other data were analyzed using a *t*-test or one-way ANOVA followed by the Student–Newman–Keuls test. For all tests, differences with $P<0.05$ were considered significant.

## 3. Results

### 3.1. 2VO-induced spatial learning impairment in mice

To test whether chronic cerebral hypoperfusion induces spatial memory deficits in mice, we occluded the bilateral common carotid arteries and assessed performance using the Morris water maze task, a hippocampus-dependent spatial learning task in which mice are required to learn to locate an escape platform (position I) in a pool of water. Consistent with studies using 2VO rats (Pappas et al., 1996; Farkas et al., 2004), 2VO mice displayed significantly longer latencies than sham-operated mice to find a platform [$F_{\text{operation}\times\text{training}}$ (5,80)=3.530, $p<0.05$; $F_{\text{operation}}$ (1,16)=41.241, $p<0.05$] in the training trials (Fig. 1A). To determine whether they were using a spatial learning strategy to locate the platform, the mice were subjected to a probe trial conducted one day after training. The sham-operated control mice spent more time searching in the target quadrant where the platform had been located during training than in each of the other quadrants (Fig. 1B). In contrast, 2VO mice spent significantly less time in the target quadrant than the sham-operated animals [$t=-6.767$, $df=16$, $p<0.05$]. In terms of swimming speed, no significant difference was found between sham-operated (11.0 ± 0.4 cm/s) and 2VO mice (10.1 ± 1.2 cm/s) [$t=0.868$, $df=16$, $p=0.406$]. Moreover, in the visible trial conducted one day after the probe trial, there was no significant difference in the time spent finding the platform between the two groups, eliminating the possibility of motivational or sensory motor deficits.

In a previous study (Murakami et al., 1997), we have demonstrated that stimulation of central cholinergic systems improves spatial memory deficits in 2VO rats. To clarify if the same is true of water maze performance, we examined the effect of tacrine on 2VO-induced spatial memory impairment in mice. Following the first series of maze training experiments, 2VO mice were given tacrine 30 min before the daily training session. As expected, tacrine (2.5 mg/kg/day, i.p.) significantly improved 2VO-induced spatial learning deficits in mice [$F(1,8)=30.402$, $p<0.05$] (Fig. 1A). In the probe trials, the 2VO mice treated with tacrine spent as much time searching in the target quadrant as the sham-operated controls, and no significant difference existed between the groups [$t=1.437$, $df=16$, $p=0.170$] (Fig. 1B).

One week after the probe trials, re-training trials in which the platform was located in the same quadrant were conducted without administering tacrine to 2VO mice. The 2VO mice showed an impaired maze performance and took longer to locate the escape platform than the sham-operated control [$F(1,16)=72.934$, $p<0.05$] (Fig. 1A).

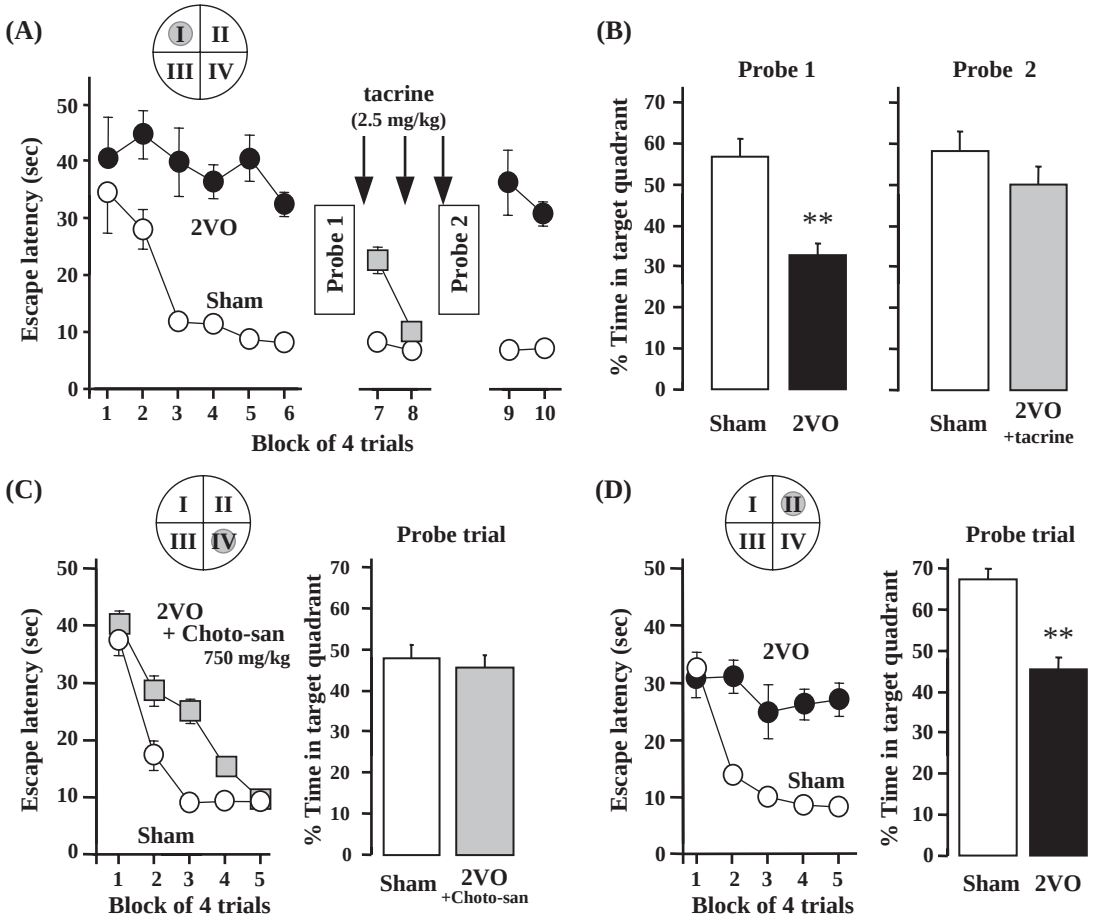


Fig. 1. The cholinesterase inhibitor tacrine and the Kampo prescription Choto-san improve two-vessel occlusion (2VO)-induced impairment of water maze performance in mice. (A) Two weeks after the 2VO operation, water maze training trials and daily drug administrations were started. Thirty minutes before each trial, 2VO mice were injected i.p. with either saline or tacrine, and sham-operated mice were injected with saline alone. Each data point indicates the mean escape latency ± S.E.M. for 9 animals. (B) Percentage of time spent in the target quadrant during a 60-s period of probe trial 1 and 2 taken at the time points indicated in Fig. 1A. (C) Choto-san administered 60 min before the testing enhanced the ability to learn the new location of the hidden platform. (D) Remembering the location of a new platform was difficult for the 2VO mice without any treatment. Each datum represents the mean ± S.E.M. for 9 animals. $^{**}p < 0.01$ compared with the sham-operated group.

After a series of experiments with tacrine, the location of the platform was changed to the side (position IV) opposite the previous site, and mice were subjected to re-training trials. 2VO mice received Choto-san (750 mg/kg, p.o.) 60 min before the daily training session. Both sham and Choto-san-treated 2VO mice learned to locate the platform [Sham: $F(4,32)=54.950$, $p<0.05$; Choto-san: $F(4,28)=32.866$, $p<0.05$], and the difference in swimming time within the target quadrant between tacrine-injected 2VO mice and sham-treated mice was not significant in the probe trial [$t=0.466$, $df=16$, $p=0.648$] (Fig. 1C).

After being left with no further treatment for 1 week, the Choto-san-pretreated 2VO mice were subjected to a 3rd round of training trials without Choto-san in which the platform location was again changed, to position II (Fig. 1D). The Choto-san-pretreated 2VO mice showed an impaired water maze performance and failed to use a spatial learning strategy to locate the escape platform [$F_{\text{group}\times\text{training}}$ (4,64)=7.694, p<0.05; $F_{\text{group}}$ (1,16)=43.367, $p<0.05$]. The percentage of time spent in the target quadrant was significantly lower in the Choto-san-pretreated 2VO mice than sham-operated control group [$t=5.676$, $df=16$, $p<0.05$].

### 3.2. Uncaria plays a major role in the ameliorating effect of Choto-san on 2VO-induced spatial learning deficit in mice

In Kampo medicine, *Uncaria* (*Uncariae Uncis cum Ramulus*) is believed to play an important role in the therapeutic action of Choto-san. Thus, we investigated the role of *Uncaria* in the effect of Choto-san on spatial learning deficits caused by 2VO in mice. First we prepared an extract of *Uncaria*-free Choto-san which contains all the herbal components of Choto-san except *Uncaria*. As shown in Fig. 2, when *Uncaria*-free Choto-san (675 mg/kg, p.o.) was administered daily for five days 1 h before the start of each training trial at doses which correspond to the amount included in Choto-san (750 mg/kg) as an extract, it did not affect the impaired spatial learning performance of 2VO mice in water maze learning trials [$F(2,19)<1$,

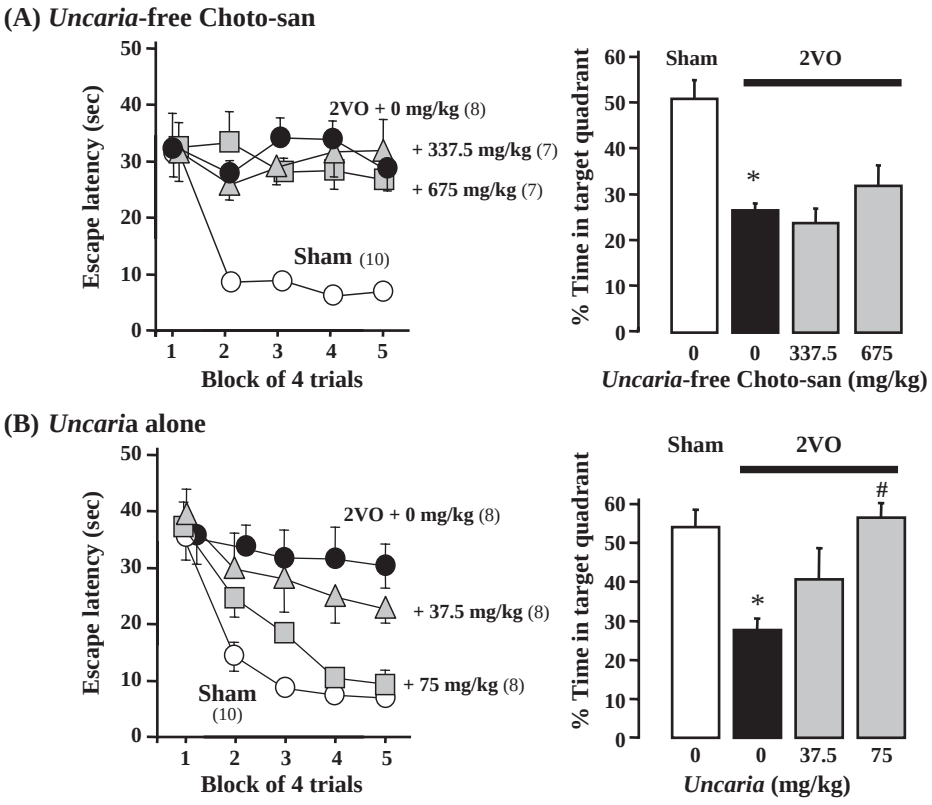


Fig. 2. *Uncaria*, a component of Choto-san, but not *Uncaria*-free Choto-san, improved 2VO-induced impairment of water maze performance in mice. Daily administration of *Uncaria*-free Choto-san (A) and *Uncaria* (B) was started 2 weeks after the 2VO operation. The water maze task was carried out 60 min after the administration. Numbers in parenthesis indicate group size. Each datum represents the mean ± S.E.M. *$p<0.05$ compared with the sham-operated group. $^{\#}p<0.05$ compared with the un-treated 2VO group.

$p=0.891$]. There was no significant difference in the swimming time in the target quadrant between vehicle-treated and *Uncaria*-free Choto-san-treated 2VO mice in the probe trial [$F(2,19)=1.679$, $p=0.213$] (Fig. 2A). In contrast, the daily administration of *Uncaria* extract (75 mg/kg, p.o.) at doses which corresponded to the amount of this plant included in Choto-san (750 mg/kg) during a training period significantly improved the learning performance in training trials [$F(2,21)=9.371$, $p<0.05$] and the animals treated with *Uncaria* extract used a spatial learning strategy to locate the platform in a probe trial [$F(2,20)=8.590$, $p<0.05$]. (Fig. 2B).

### *3.3. Involvement of central cholinergic systems in the beneficial effects of tacrine and Choto-san on 2VO-induced spatial learning impairment in mice*

In our previous study, systemic administration of tacrine reversed the 2VO-induced spatial cognitive impairment in rats and the effect was in part mediated by the stimulation of muscarinic $M_1$ receptors by acetylcholine (Murakami et al., 2000). We, thus, examined if the $M_1$ receptor is involved in the ameliorating effect of Choto-san in 2VO mice. Fig. 3A showed that the escape latency differed significantly between the group administered Choto-san (750 mg/kg, p.o.) and that given Choto-san combined with pirenzepine (100 mg/kg, i.p.), a muscarinic $M_1$ receptor antagonist [$F_{\text{training}\times\text{treatment}}(4,84)=3.651$, $p<0.05$; $F_{\text{treatment}}(1,21)=31.152$, $p<0.05$]. In the probe trial, the 2VO mice treated with Choto-san and pirenzepine showed a significant decrease in the amount of time spent in the target quadrant compared with those administered Choto-san alone [$t=6.422$, $df=21$, $p<0.05$]. The ameliorating effects of tacrine (2.5 mg/kg/day, i.p.) on the impaired spatial learning performance of 2VO mice in training and probe trials were significantly blocked by co-administraiton of pirenzepine [$F_{\text{training}\times\text{treatment}}(4,72)=3.559$, $p<0.05$; $F_{\text{training}}(1,18)=15.014$, $p<0.05$; probe: $t=3.833$, $df=18$, $p<0.05$] (Fig. 3B).

### *3.4. 2VO-induced changes in acetylcholine content of the brain*

The central cholinergic systems play important roles in the regulation of cerebral circulation (Scremin et al., 1973), and cognitive function such as memory and attention (Whishaw and Tomie, 1987; Muir et al., 1993). Moreover, it was demonstrated that 2VO reduced the level of ACh in rat brain (Ni et al., 1995a). On the basis of these findings, we measured brain ACh levels and elucidated the effects of Choto-san on central cholinergic system using the mice that were trained in the water maze test.

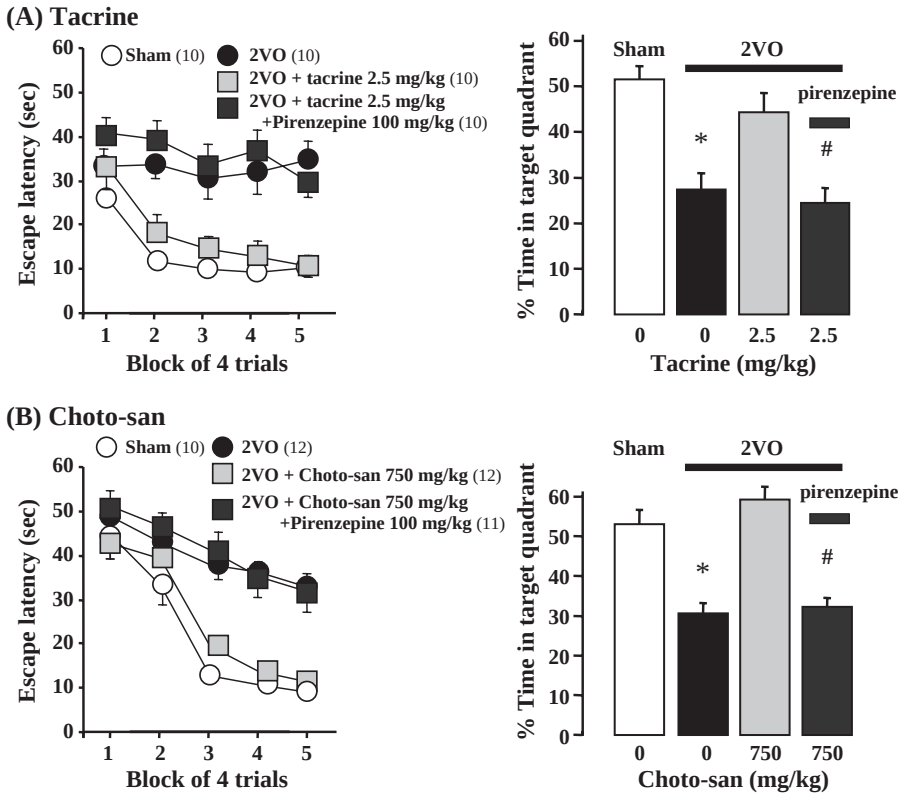


Fig. 3. Effect of pirenzepine, a selective muscarinic $M_1$ antagonist, on tacrine- and Choto-san-induced amelioration of impaired water maze performance in 2VO mice. (A) Pirenzepine (100 mg/kg) was injected i.p. 30 min before the start of each learning trial. Tacrine was given i.p. immediately after pirenzepine. (B) Choto-san was administered p.o. 30 min before pirenzepine. Number of animals used is given in parenthesis. Each datum represents the mean ± S.E.M. *$p < 0.05$ compared with the sham-operated group. #$p < 0.05$ compared with the tacrine- or Choto-san-treated 2VO group.

As shown in Fig. 4, a one-way analysis of variance revealed an overall group difference in ACh levels in the cerebral cortex and hippocampus [cerebral cortex: $F(5,35)=3.852$, $p<0.05$; hippocampus: $F(5,35)=5.127$; $p<0.05$]. Post hoc analyses showed that ACh levels were significantly decreased in 2VO mice relative to the sham-

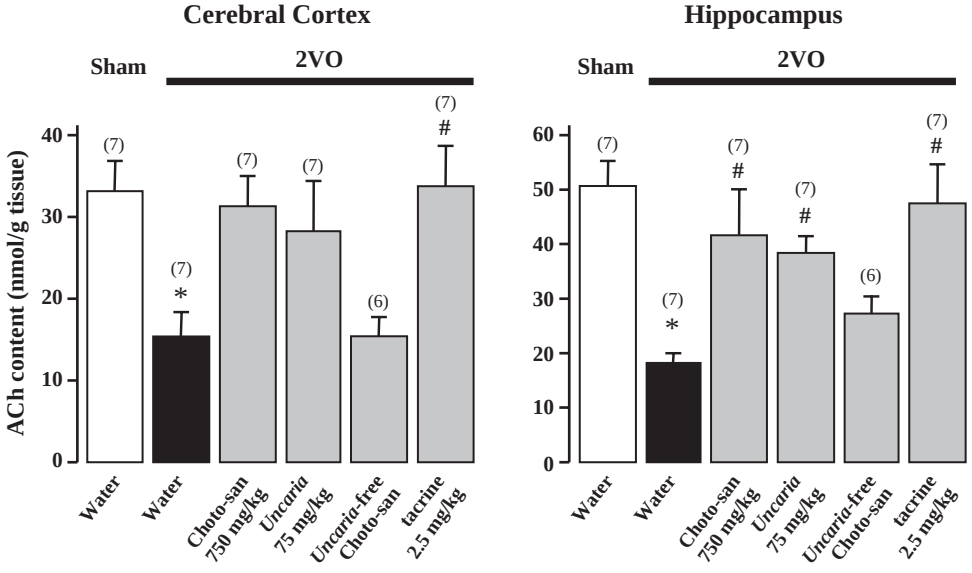


Fig. 4. Effect of test drugs on ACh levels in the cerebral cortex and hippocampus of 2VO mice. Test drugs were administered daily for seven days to 2VO mice, and the animals were decapitated 60 min after the last administration. ACh levels in the cerebral cortex and hippocampus were measured as described in the text. Each column represents the mean ± S.E.M of n (in parenthesis) observations. *$p < 0.05$ compared with the sham-operated group. #$p < 0.05$ compared with the untreated 2VO group.

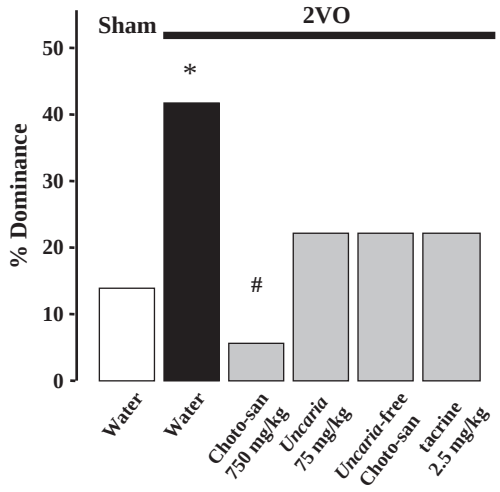


Fig. 5. Effect of test drugs on the assertive behavior induced by 2VO in mice. Test drugs were administered daily for seven days prior to the experiments. The assertive behavior was measured 2 h after the last administration using the tube-dominance test. Each column represents the percentage of dominance in 36 cases (9 animals × 4 times). *$p<0.05$ compared with the sham-operated group. #$p<0.05$ compared with the untreated 2VO group.

operated control group in both regions, and that the hippocampal ACh level in 2VO mice was significantly increased by treatment with Choto-san, *Uncaria*, and tacrine, whereas the cortical ACh levels of Choto-san- and *Uncaria*-treated 2VO rats were slightly different from those of 2VO control [Choto-san: $p=0.067$; *Uncaria*: $p=0.197$]. No difference occurred between *Uncaria*-free Choto-san-treated 2VO mice and 2VO mice [cerebral cortex: $p=0.970$; hippocampus: $p=0.252$].

### 3.5. Choto-san prevented 2VO-induced assertive behavior in the tube-dominance test

Agitation/aggression is the most common behavioral disturbance found in patents with dementia (Lyketsos et al., 2000). Thus, we next examined using a tube-dominance test whether 2VO mice showed aggressive behavior. 2VO mice showed more assertive responses than sham-operated mice [$\chi^2=5.608$, $p<0.05$] (Fig. 5). In contrast, 2VO mice administered with Choto-san over seven days showed a decrease in dominance behavior [$\chi^2=11.089$, $p<0.05$]. When we administered *Uncaria*, *Uncaria*-free Choto-san, or tacrine, the rates of assertive behavior were not significantly changed [$\chi^2=2.30$, $p=0.129$].

## 4. Discussion

The present study demonstrates that mice with chronic cerebral hypoperfusion induced by 2VO show persistent learning impairment, and that this animal model may be useful not only for studying the pathophysiology of learning and memory deficits in human dementia with cerebral circulation impairment but also for assessing the nootropic action of drugs. Our findings demonstrate that Choto-san, a Kampo prescription, improves 2VO-induced spatial learning disabilities, via the stimulation of muscarinic $M_1$ receptors, and that the *Uncaria* in Choto-san plays an important role in this beneficial effect.

### 4.1. 2VO mice as a model of learning impairment due to cerebral hypoperfusion

A variety of animal models of stroke have been established to study the pathophysiology of brain ischemia, and to evaluate the anti-ischemic (neuroprotective) or nootropic activity of chemicals. Most prior studies have concentrated on transient cerebral ischemia models. However, there are conflicting reports on whether transient ischemia causes cognitive impairment (Auer et al., 1989; Hagan and Beaughard, 1990; Green et al., 1992, 1995; Shuaib et al., 1995) or whether the impairment is limited to temporary symptoms (Volpe et al., 1984; Davis et al., 1986; Gionet et al., 1991; Corbett et al., 1992; Sakai et al., 1996). The discrepancy may be due to methodological factors such as the conditions used to induce ischemia and behavioral tasks used to evaluate learning and memory ability. On the other hand, a permanent complete ischemia exacerbates not only cognitive abilities but also physical functions over a long period (Okada et al., 1995; Aspey et al., 1998; Yonemori et al., 1999; Hunter et al., 2000), indicating that deficits in the sensory motor system of the model are a disadvantage when estimating learning ability. In contrast, a chronic hypoperfusion model in rats and gerbils reportedly exhibits a long-lasting impairment of learning ability, although there are conflicting reports on the histopathological damage found in this animal model (Kudo et al., 1990; de la Torre et al., 1992; Ni et al., 1994; Ohta et al., 1997; Sekhon et al., 1997). The present study indicated that mice with chronic cerebral hypoperfusion caused by 2VO exhibit learning impairments in a water maze task without marked histological alterations in the hippocampus and cerebral white matter, regions vulnerable to ischemia (Ni et al., 1995a; Pantoni et al., 1996; Nanri et al., 1998a). Moreover, a persistent impairment of spatial learning in 2VO mice was revealed by performing re-learning trials in which the location of the platform was changed to another quadrant of the pool. These findings also support the idea that the 2VO mouse is a useful model of dementia resulting from interference with cerebral blood flow, and is available for assessing therapeutic potential and/or exploring the mechanism(s) of action of putative anti-dementia drugs.

### 4.2. Choto-san reversibly improves learning in 2VO mice

In our previous study, Choto-san exhibited a protective effect on transient cerebral ischemia-induced spatial learning impairment in mice (Watanabe et al., 2003). Very little

information, however, is available on the effects of Choto-san on learning and memory performance impaired by cerebral ischemia in experimental animals (Yuzurihara et al., 1999). In the present study, we have demonstrated that post-ischemic administrations of Choto-san and tacrine significantly improve spatial learning performance impaired by chronic cerebral hypoperfusion in mice. These findings not only indicate the therapeutic potential of this Kampo formula, but also provide experimental support for the clinical use of Choto-san in the treatment of cerebrovascular dementia (Terasawa et al., 1997).

Interestingly, when the tacrine- and Choto-san-pretreated 2VO mice were left with no further treatment for 1 week, they showed a significantly impaired learning performance compared to the sham-operated control animals in the re-learning test which was conducted without administration of these drugs. These findings indicate that the ameliorating effect of Choto-san and tacrine on the spatial learning performance of 2VO animals is reversible, and depends on the administration of these drugs during the maze learning period. The reversible effect of tacrine found in 2VO mice is consistent with the data we obtained using 2VO rats (Murakami et al., 2000).

### 4.3. The role of Uncaria in Choto-san-induced improvement of learning deficits in 2VO mice

The *Uncaria* extract dose-dependently ameliorated the effect of 2VO on maze learning performance at a dose range corresponding to the amount of *Uncaria* included in Choto-san, whereas the *Uncaria*-free Choto-san extract had no effect. These results suggest that *Uncaria* plays a key role in the beneficial effect of Choto-san on learning and memory impairment caused by chronic hypoperfusion. Previous studies from this and other laboratories have indicated that *Uncaria* takes part in a protective effect on ischemia-induced learning deficits, neuronal cell death, and oxidative stress (Watanabe et al., 2003; Na et al., 2004; Yokoyama et al., 2004). Moreover, we previously showed that *Uncaria* plays an important role in the anti-hypertensive effect of Choto-san in spontaneous hypertensive rats (Zhao et al., 2002). Considering that people with high blood pressure are at high risk of developing vascular dementia, *Uncaria* in Choto-san would be useful not only for prevention but also treatment of vascular dementia.

The present results do not imply that the herbal components other than *Uncaria* in Choto-san are dispensable for the treatment of cerebrovascular dementia, because Choto-san but not *Uncaria* significantly attenuated the aggressiveness induced by 2VO. The herbal constituents responsible for the Choto-san-induced suppression of aggressiveness remain unclear but a speculative explanation is that chemical reactions between *Uncaria* constituents and other herbal constituents may occur during decoction and results in compound(s) effective for suppression of aggressiveness. Nevertheless, considering the present data that tacrine failed to attenuate the aggressiveness in 2VO mice, Choto-san may be more useful than *Uncaria* alone or tacrine in the treatment of cerebrovascular disease.

### 4.4. Mechanisms underlying the beneficial effect of Choto-san

In this series of experiments, we used the cholinesterase inhibitor tacrine as a reference drug, and demonstrated that it ameliorated learning deficits induced by 2VO in a manner that was blocked by the selective muscarinic $M_1$ receptor antagonist pirenzepine. The pirenzepine-reversible effect of tacrine on the 2VO-induced impairment of spatial learning performance is consistent with our previous reports (Murakami et al., 2000), and indicates that the stimulation of muscarinic $M_1$ receptors by enhancing central cholinergic systems can improve spatial learning deficits caused by 2VO in rodents. Moreover, it is worth noting that the Choto-san-induced amelioration of spatial learning impairment in 2VO mice was also attenuated by the $M_1$ receptor antagonist. Thus it is very likely that Choto-san, as well as tacrine, is able to enhance cholinergic systems in the brain.

Several factors may explain the mechanism(s) underlying the beneficial effect of Choto-san in 2VO mice. First, some component(s) of Choto-san may act as a $M_1$ receptor agonist and improve learning and memory deficits caused by cerebral hypoperfusion. This possibility, however, seems remote, because several lines of evidence indicate that $M_1$ receptor agonists induce a decrease in brain ACh content (Ogane et al., 1990; Enz et al., 1993), whereas in the present study, Choto-san reversed the decline in brain ACh levels evoked by 2VO treatment. Second, the constituent(s) of Choto-san may be able to stimulate indirectly the $M_1$ receptor via an elevation of the extracellular ACh level in the brain. In this study, Choto-san increased ACh levels in the brain of 2VO mice. Together, it is likely that daily treatment with Choto-san during a maze learning period inhibits the activity of cholinesterase and/or enhances the activity of choline acetyl transferase. Third, neuronal systems other than the central cholinergic system may be involved in the action of Choto-san in mice with cerebral hypoperfusion. In a previous study, the effect of Choto-san on the ischemia-induced impairment of a step-through type passive avoidance performance was blocked by a serotonin 1A antagonist (Yuzurihara et al., 1999). Moreover, stimulation of the serotonin 1A receptor appears to increase the amount of ACh released in the brain (Bianchi et al., 1990; Siniscalchi et al., 1990). Thus, the present results raise the possibility that the enhancement of cholinergic activity via serotonergic systems is involved in the ameliorating effect of Choto-san on spatial learning deficits in 2VO mice. Nevertheless, these explanations require experimental support.

## 5. Conclusion

The present findings demonstrate that Choto-san, as well as tacrine, improves a spatial learning impairment induced by cerebral hypoperfusion and that the beneficial effect of Choto-san is mainly attributable to *Uncaria* and is mediated by stimulation of the muscarinic $M_1$ receptor. The present study provides pharmacological evidence to support the clinical finding that Choto-san is useful for the treatment of vascular dementia.

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