Preventive Effect of Chotosan, a Kampo Medicine, on Transient Ischemia-
Induced Learning Deficit Is Mediated by Stimulation of Muscarinic M₁
But Not Nicotinic Receptor

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We have previously shown using a water maze task that transient 2 vessel occlusion (T2VO) induced learning
deficit in mice and that the deficit was prevented by pre-treatment of mice with chotosan, a Kampo prescription.
In this study, we investigated the mechanism underlying the preventive effect of chotosan on T2VO-induced
learning deficit. Chotosan administration 1 h before T2VO operation prevented learning impairment. The extract
of Uncaria, a major constituent of chotosan, also had a protective effect on learning impairment in T2VO
mice, whereas Uncaria-free chotosan had no beneficial effect on maze performance of T2VO mice. The ameliorative
effect of chotosan was blocked by pirenzepine, a muscarinic M₁ antagonist, but not by mecamylamine, a
nicotinic receptor antagonist. Acetylcholine (ACh) content in the hippocampus of T2VO mice was significantly
lower than that in the hippocampus of sham-operated control mice. Chotosan and Uncaria administration attenu-
ated T2VO-induced reduction of ACh levels in the brain. These results suggest that the preventive effect of
chotosan on transient ischemia-induced learning impairment is mainly attributable to the effect of Uncaria and
that the ameliorative effect is mediated by stimulation of muscarinic M₁ receptor.

Key words chotosan; transient cerebral ischemia; spatial memory; muscarinic M₁ receptor; nicotinic receptor; mouse

People with high blood pressure are at high risk for developing vascular dementia. Thus, the primary treatment to
prevent further worsening of vascular dementia is to control the underlying disease such as hypertension.

Chotosan is a Kampo (traditional medicine of Japan) prescription used for treatment of relatively elderly patients with
chronic headache or dizziness associated with hypertension. Recently, Terasawa et al. have reported the effectiveness of
chotosan in the treatment of cognitive impairments of patients with stroke or hypertension. In previous in vivo studies,
we demonstrated that transient ischemia-induced learning deficit was prevented by administration of chotosan and Uncaria,
a major constituent of chotosan. Moreover, in vitro studies have suggested that the preventive effects of choto-
san are in part due to its phenolic compounds with free radical scavenging activity and indole alkaloid components with
antioxidant activity, since free radical generation and excessive activation of N-methyl-D-aspartate type glutamate
receptors are involved in neuronal cell death caused by ischemia. However, less information is available on the
mechanisms by which chotosan exerts a protective effect against cerebral ischemia in vivo.

Several lines of evidence indicate that cholinesterase inhibitors increase cerebral blood flow and exert a protective
action against ischemia-induced learning deficit and neuronal cell death. Muscarinic receptors appear to be implicated
in inhibition of delayed neuronal death after transient ischemia probably via increasing neurotrophic factors such
as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Moreover, stimulation of nicotinic α7 recep-
tors reportedly shows a similar protective effect on ischemia-induced neuronal injury. Thus, cholinergic tone
may play an important role in the protection from ischemic injury. In the present study, we investigated whether chotosan
exerts a protective effect on ischemia-induced insults through cholinergic systems.

MATERIALS AND METHODS

Animals Male ICR mice (Japan SLC Inc., Hamamatsu, Japan) were housed in the laboratory animal room main-
tained 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 148 mice were used for the ex-
periments. 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 Ani-
mal Use and Care Committee of Toyama Medical and Pharmaceutical University.

Transient Ischemia Operation The animals, aged 8 weeks, 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 occluded with aneurysm clips for 20 min and hypotension
was produced by blood withdrawal (0.3 ml) from the tail during ischemic operation. The animals that received the same
surgical operation without carotid clamping and bleeding served as the sham-operated controls.

Water Maze Task Water maze performance of mice was tested using a 1.1 m diameter circular pool according to our
previous report. Two days after the operation, mice were subjected to a visible trial (Visible 1) of water maze in which the
platform was made visible 1 cm above the water surface. Training trials were performed daily for 5 days 1 day after the
visible trial. Mice received 4 trials per day on training trials. Each trial consisted of placing the mouse in the pool at one of
4 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 during a 60-s period, 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 (escape latency), 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 4 trials before being used for statistical analysis. One day after the acquisition trials for 5 d, 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 cue trial was again performed to check the vision of all mice.

**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). The homogenate was centrifuged at 10000 × g for 0.9 s, allowing the mouse to swim to the hidden transparent platform (7 cm in diameter). The homogenate 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 0.45 mM Na2EDTA with a Polytron homogenizer (PT-10, Kinematica, Switzerland). The homogenate was centrifuged at 10000 × 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).

**Drugs**

Daily dosage (28 g) of chotosan 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 were deposited in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University. To prepare the chotosan 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 decanted for 1 h, with *Uncaria* added for the last 15 min. An extract prepared without adding *Uncaria* was used as *Uncaria*-free chotosan. To prepare the *Uncaria* extract, *Uncariae Uncis cum Ramulus* was mixed with the decoction of distilled water, and decoced for 15 min. After filtration, each extract was freeze-dried. The yields of the extract of chotosan, *Uncaria*-free chotosan 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 chotosan extract was dissolved in distilled water and orally administered to mice 60 min before the T2VO operation. Tacrine (9-amino-1,2,3,4-tetrahydroacridine HCl; Sigma Chemical Co., St. Louis, MO, U.S.A.), a reference drug, was dissolved in physiological saline and injected intraperitoneally 30 min before the ligation.

**Statistical Analysis**

All results were expressed as the mean±S.E.M. The data obtained in the water maze task were analyzed by one-way or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons among different groups. The neurochemical data were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test. For all tests, differences with p<0.05 were considered significant.

**RESULTS**

**Water Maze Performance**

Figure 1 shows the effects of chotosan and tacrine on water maze performance of T2VO mice. In the visible trials conducted before (Visible 1) and after training trials (Visible 2) (Fig. 1A), no differences in the escape latency were found among the groups [Visible 1: F(4, 41)=0.0632, p=0.992; Visible 2: F(4, 41)=2.099, p=0.098], indicating that pretreatment with test drugs had no influence on motivation or sensory motor system in vehicle control T2VO mice. In sham-operated mice, latencies to escape onto the platform were significantly shorter than those of the sham-operated mice. In the visible trials conducted before (Visible 1) and after training trials (Visible 2) (Fig. 1A), no differences in the escape latency were found among the groups [Visible 1: F(4, 41)=0.0632, p=0.992; Visible 2: F(4, 41)=2.099, p=0.098], indicating that pretreatment with test drugs had no influence on motivation or sensory motor system in vehicle control T2VO mice. In sham-operated mice, latencies to escape onto the platform were significantly shortened by daily training [Ftraining(4, 36)=32.53, p<0.001]. The escape latencies of vehicle control T2VO mice were also reduced by training [Ftraining(4, 36)=7.068, p<0.001], but this animal group needed significantly longer time to find the hidden platform than the sham-operated group [Foperation(1, 18)=118.108, p<0.001]. Administration of chotosan (750 mg/kg) 1 h before T2VO operation prevented learning disturbance caused by T2VO in the water maze task [Ftreatment(1, 17)=47.067, p<0.001] (Fig. 1A).

In the probe test conducted 1 d after 5 d of training (Fig. 1B), swimming time of the T2VO control mice in the target quadrant where the platform had been located during training was significantly shorter than that of the sham-operated animals [F(1, 18)=20.041, p<0.001] (Fig. 1B). Pretreatment of T2VO mice with tacrine, a cholinesterase inhibitor, significantly prolonged swimming time in the target quadrant [F(1, 16)=27.343, p<0.001]. Chotosan pretreatment, as well as tacrine, significantly and dose-dependently reversed T2VO-induced decrease in swimming time in the target quadrant [F(2, 25)=6.407, p<0.01].

On the other hand, pretreatment with *Uncaria*-free chotosan had no effect on learning performance of T2VO mice in the training test [Ftreatment(1, 17)=3.307, p=0.09] (Fig. 2A) or the swimming time in the probe test [F(1, 17)=3.758, p=0.07] (Fig. 2B), whereas *Uncaria* extract, when given at the doses which were approximately equivalent to the amount of this herb included in chotosan, dose-dependently reduced the escape latency in the training test [Ftreatment(2, 27)=7.116, p<0.01] and prolonged the swimming time in the target quadrant (p<0.05) (Figs. 2C, D).

We, next, examined if the central cholinergic system is involved in the effect of chotosan, since our previous study demonstrated that this prescription enhanced cholinergic activity in a chronic cerebral hypoperfusion model of mice. When pirenzepine, a muscarinic M1 antagonist, was injected 30 min after chotosan administration, significantly blocked
the preventive effect of chitosan on the escape latency to the hidden platform in the training test $[F_{\text{treatment}}(1, 17) \times H_{11005}^{1}(16.462), p_{H_{11021}}^{0.001}]$ (Fig. 3A). Pirenzepine also attenuated the ameliorative effect of *Uncaria* on water maze performance $[F_{\text{treatment}}(1, 18) = 11.925, p_{H_{11021}}^{0.01}]$ (Fig. 3C). Similarly, the effect of tacrine on water maze performance of T2VO mice was inhibited by co-administration of pirenzepine $[F_{\text{treatment}}(1, 16) = 28.700, p_{H_{11021}}^{0.001}]$ (Fig. 3E). The data obtained in the probe test showed that pirenzepine significantly decreased the time spent in the target quadrant in chitosan-pretreated T2VO mice (Figs. 3B, D, F). In contrast to the effect of pirenzepine, mecamylamine, a neuronal nicotinic receptor antagonist, failed to reverse the effects of tacrine $[F_{\text{treatment}}(1, 17) = 0.617, p_{H_{11005}}^{0.443}]$ and chitosan $[F_{\text{treatment}}(1, 17) = 0.151, p_{H_{11005}}^{0.703}]$ on learning performance in the training test (Fig. 4A). As shown in Fig. 4B, mecamylamine treatment had no effect on chitosan- or tacrine-induced prolongation of swimming time in the target quadrant in the probe test.

**Changes in ACh Levels in the Hippocampus** Effects of T2VO on ACh levels in the hippocampus were examined 1 d after completion of behavioral studies. As shown in Table 1,
there is a significant difference among the groups \( F(3, 22) = 6.112, \ p < 0.01 \). Post hoc comparisons showed that T2VO mice had a significantly reduced level of ACh in the hippocampus \( (p < 0.01) \). Pretreatment with chotosan, but not with Uncaria-free chotosan, significantly attenuated the reduction of hippocampal ACh level caused by T2VO. Uncaria extract, as well as chotosan, significantly prevented the T2VO-induced decrease in hippocampal ACh levels (Table 2). Treatment with pirenzepine significantly abolished the effects of chotosan, Uncaria and tacrine on the hippocampal ACh level in T2VO mice. In contrast, pretreatment with mecamylamine had no effect on reversal of hippocampal ACh level by chotosan and tacrine in T2VO animals (Table 3).

![Fig. 4. Effect of Mecamylamine, a Nicotinic Antagonist, on Tacrine- and Chotosan-Induced Amelioration of Impaired Water Maze Performance in T2VO Mice](image)

**Table 1. Effects of Chotosan, Uncaria, and Uncaria-Free Chotosan on T2VO-Induced Changes in Acetylcholine Level in Mouse Hippocampus**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>ACh level (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>42.8 ± 7.1</td>
</tr>
<tr>
<td>T2VO control</td>
<td>14.0 ± 2.5*</td>
</tr>
<tr>
<td>T2VO + chotosan (750)</td>
<td>33.9 ± 6.0*</td>
</tr>
<tr>
<td>T2VO + Uncaria-free chotosan (675)</td>
<td>22.4 ± 4.7</td>
</tr>
</tbody>
</table>

The extracts were orally administered 60 min before T2VO. Each datum represents the mean ± S.E.M. for 6—7 animals. \( \ast \ p < 0.05 \) compared with the sham-operated group.

![Fig. 3. Effect of Pirenzepine, a Selective Muscarinic M₁ Antagonist, on Tacrine-, Chotosan-, and Uncaria-Induced Amelioration of Impaired Water Maze Performance in T2VO Mice](image)

**Table 2. Effect of Pirenzepine on Chotosan-, Uncaria-, and Tacrine-Induced Preventive Effect on ACh Decrease in T2VO Mice Brain**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>ACh level (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>53.8 ± 8.7</td>
</tr>
<tr>
<td>T2VO</td>
<td>21.0 ± 2.3*</td>
</tr>
<tr>
<td>T2VO + chotosan (750)</td>
<td>38.4 ± 2.9*</td>
</tr>
<tr>
<td>T2VO + chitosan + pirenzepine (100)</td>
<td>22.7 ± 3.3*</td>
</tr>
<tr>
<td>T2VO + Uncaria (75)</td>
<td>43.2 ± 6.4*</td>
</tr>
<tr>
<td>T2VO + Uncaria + pirenzepine (100)</td>
<td>24.4 ± 7.9*</td>
</tr>
<tr>
<td>T2VO + tacrine (2.5)</td>
<td>31.9 ± 3.3*</td>
</tr>
<tr>
<td>T2VO + tacrine + pirenzepine (100)</td>
<td>17.8 ± 3.4*</td>
</tr>
</tbody>
</table>

The extracts were orally administered 60 min before ischemia. Tacrine and pirenzepine were intraperitoneally injected 30 min before ischemia. Each datum represents the mean ± S.E.M. for 7—10 mice. \( \ast \ p < 0.05 \) compared with the sham-operated group. \( \ast \ p < 0.05 \) compared with the T2VO control group. \( \ast \ p < 0.05 \) compared with the chotosan treated T2VO group. \( \ast \ p < 0.05 \) compared with the Uncaria treated T2VO group.

![Fig. 4. Effect of Mecamylamine, a Nicotinic Antagonist, on Tacrine- and Chotosan-Induced Amelioration of Impaired Water Maze Performance in T2VO Mice](image)
Table 3. Effect of Mecamylamine on Chotosan and Tacrine on Acetylcholine Content in the T2VO Mice Brain

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>ACh level (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>52.8±8.9</td>
</tr>
<tr>
<td>T2VO control</td>
<td>18.6±2.8*</td>
</tr>
<tr>
<td>T2VO+chotosan (750)</td>
<td>51.1±11.1†</td>
</tr>
<tr>
<td>T2VO+chitosan+mecamylamine (10)</td>
<td>36.8±8.2</td>
</tr>
<tr>
<td>T2VO+chitosan (2.5)</td>
<td>49.8±7.4‡</td>
</tr>
<tr>
<td>T2VO+chitosan+mecamylamine (10)</td>
<td>41.1±4.8</td>
</tr>
</tbody>
</table>

Chitosan was orally administered 60 min before T2VO. Tacrine and mecamylamine were intraperitoneally injected 30 min before T2VO. Each datum represents the mean±S.E.M. for 5 animals. *p<0.05 compared with the sham-operated group. †p=0.05 compared with the untreated T2VO group.

DISCUSSION

This study has demonstrated that chitosan prevents T2VO-induced spatial learning deficits and reduction of the hippocampal acetylcholine level in mice and that Uncaria, a major component herb of chitosan, plays an important role in the effects of chitosan. Moreover, the present results have suggested that muscarinic M1 receptors but not nicotinic receptors are involved in the preventive effect of chitosan and Uncaria extract.

Pretreatment with chitosan, as well as with a cholinesterase inhibitor tacrine, dose-dependently attenuated T2VO-induced spatial cognitive deficits in a water maze task. It should be noted that when examined the effects of Uncaria extract and Uncaria-free chitosan at the doses that are approximately relevant to the amount included in chitosan, the Uncaria extract but not Uncaria-free chitosan prevented impairment of water maze learning performance caused by T2VO. These findings are consistent with previous reports from this laboratory and others and further support the idea that the preventive effect of chitosan is mainly attributable to constituents included in the Uncaria extract.

A line of evidence indicates that the cholinergic system in the brain plays an important role in learning and memory and that hypofunction of the central cholinergic system causes learning deficits. Moreover, many studies have demonstrated that ischemia causes a reduction of memory and judgment that is associated with the cholinergic dysfunction in affected brain areas and that ischemic insults decreases in ACh level in the affected regions of the brain. Thus, in animals subjected to transient ischemia, an impairment of spatial cognitive performance in a water maze task is likely attributed to dysfunction of the central cholinergic system due to disturbance of energy metabolism and hypoxia in the ischemic brain. Consistent with previous reports from this and other laboratories, we found that transient ischemia caused a significant reduction of the ACh level in the hippocampus and that pretreatment with a cholinesterase inhibitor tacrine produced a protective effect not only on learning deficits but also on the reduction of the hippocampal ACh level in T2VO mice. These findings raise the possibility that tacrine protects the brain against transient ischemic insults by augmenting ACh level in the brain and thereby stimulating at least muscarinic M1 receptors since the effects of tacrine were blocked by pirenzepine, a selective muscarinic M1 receptor antagonist, but not by mecamylamine, a nicotinic receptor antagonist.

It is of interest to note that pretreatment with chitosan and Uncaria extract, as well as with tacrine, exerted the protective action against T2VO-induced decrease in the hippocampal ACh level and that the effects was abolished by pirenzepine but not by mecamylamine. Previous studies demonstrated that the neuroprotective effect of chitosan was in part due to its phenolic compounds with free radical scavenging activity and oxindole alkaloid components, such as rhynchophylline and isorhynchophylline, with an anti-excitotoxic activity. Thus, in addition to these pharmacological properties, it is likely that the protective action of chitosan and Uncaria extract on dysfunction of the central cholinergic systems caused by brain ischemia involves stimulation of muscarinic M1 receptors.

The neuronal mechanism by which tacrine and chitosan exert preventive effects on transient ischemia-induced learning deficit and hippocampal ACh reduction remains unclear. Lines of evidence indicate that a total volume of cerebral blood flow per se is maintained at a stable level within a narrow ranges by the autoregulation mechanism, whereas regional cerebral blood flow can be increased not only by enhancement of glucose metabolism in regional neurons but also by activity of several neuronal systems, particularly a cholinergic system originating from the basal forebrain. Moreover, it has been demonstrated that the cholinergic systems produce vasodilative action in the cerebral cortex and hippocampus, the brain areas quite vulnerable to ischemia. Considering M1 muscarinic receptor antagonist-reversible properties of the pharmacological action of these drugs observed in present and previous studies, one may infer that tacrine and chitosan improve regional cerebral blood flow during ischemia by enhancing a central cholinergic system, leading to a reduction of ischemic brain damage. However, this possibility may be little since cholinergic vasodilation in the cortex and hippocampus involves muscarinic and nicotinic receptor stimulation. Nevertheless, the mechanism underlying the protective actions of chitosan, Uncaria extract, and tacrine on T2VO-induced neuronal damage needs further investigation.

In previous in vitro studies, rhynchophylline and isorhynchophylline, oxindole alkaloid components of Uncaria species, exhibited antagonistic properties for NMDA type glutamate receptors, muscarinic receptors and 5-HT2 receptors in a receptor expression model of Xenopus oocytes. Moreover, these alkaloids, as well as pirenzepine, the NMDA antagonist (±)-2-amino-5-phosphono-valeric acid, and the 5-HT2 antagonist ketanserin, protected in vitro ischemia-induced neuronal damage in the hippocampus, suggesting that these alkaloids exerted their protective action against in vitro ischemia-induced neuronal damage via blockade of NMDA, M1, and 5-HT2 receptors. Therefore, the apparent agonistic properties of chitosan and the Uncaria extract on central cholinergic systems found in this study are in contrast to the action of Uncaria alkaloids observed in the aforementioned in vitro studies. The exact reason for this discrepancy remains unclear. However, considering the previous report that rhynchophylline administered orally before ischemic operation attenuated transient ischemia-induced spatial cognitive performance in mice, the metabolite(s) of the Uncaria alkaloid constituent(s) may play a role in apparent enhancement of central cholinergic systems in the brain.
In conclusion, chotosan exerts a protective effect on transient ischemia-induced spatial cognitive impairment and neuronal dysfunction in mice. The effects of chotosan involve stimulation of muscarinic M₁ receptors in the brain. Considering our previous findings that chotosan has an anti-hypertension effect and improved learning performance impaired by permanent occlusion of common carotid arteries in mice, the present findings suggest that this prescription is beneficial for preventing against and curing cerebrovascular dementia.

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