J. Trad. Med. 23, 117－131, 2006

Medical and Pharmaceutical Society for WAKAN-YAKU

Review

Chotosan and cerebrovascular disorders: Clinical and experimental studies

Yutaka SHIMADA,[*a,b)] Hirozo GOTO,[a,b)] and Katsutoshi TERASAWA[b,c)]

[a)]*Department of Japanese Oriental Medicine, Faculty of Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.* [b)]*21st Century COE Program, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.* [c)]*Department of Japanese Oriental Medicine, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan.* *(Accepted September 12, 2006.)*

We reviewed our prior clinical and experimental studies about the effects of chotosan, a Kampo formula, on cerebrovascular disorders. A double-blind randomized controlled trial demonstrated that chotosan was effective on vascular dementia. Chotosan improved microcirculation and hemorheological factors in patients with asymptomatic cerebral infarction. This formula inhibited the elevation of blood pressure, protected endothelial function, and prolonged the life span of stroke-prone spontaneously hypertensive rats. The phenolic fraction of *Uncaria sinensis* (US), a main medicinal plant of chotosan, had endothelium-dependent relaxation effect, and its alkaloid fraction possessed endothelium-independent relaxation effect. It was revealed that US had neuroprotective effects on glutamate- and NO donor-induced neuronal death in cultured neurons. Chotosan and US had protective effects on delayed neuronal death after transient forebrain ischemia in gerbils, and enhanced superoxide anion and hydroxyl radical scavenging activities and catalase activity in the brain. All of these results suggest that chotosan has such multiple pharmacological actions as improving effect on microcirculation, protective effect on endothelial function, and neuroprotective effect, and this formula is useful for prevention of the development of cerebrovascular disorders.

Key words chotosan, *Uncaria sinensis*, vascular dementia, microcirculation, endothelial protection, neuroprotection.

Abbreviations HDS-R, revised version of Hasegawa's dementia scale; DEA, maximum diameter of a column of intravascular erythrocyte aggregation; NO, nitric oxide; NO_2^-/NO_3^-, nitrite/nitrate; LPO, lipid peroxide; SHR-SP, stroke-prone spontaneously hypertensive rat; NE, norepinephrine; Ach, acetylcholine; SNP, sodium nitroprusside; US, *Uncaria sinensis*; USE, *Uncaria sinensis* extract; USE-W, water elute of *Uncaria sinensis* extract; USE-P, phenolic fraction of *Uncaria sinensis* extract; USE-A, alkaloid fraction of *Uncaria sinensis* extract; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; L-NAME, N^G-nitro-l-arginine methylester; SOD, superoxide dismutase; SHR, spontaneously hypertensive rat; NMDA, *N*-methyl-D-aspartate; DMSO, dimethylsulphoxide; SIN-1, 3-morpholinosydnomimine; i/rp, ischemia/reperfusion; CSE, chotosan extract; CAT, catalase; GSH-Px, glutathione peroxidase; $O_2^{-}\cdot$, superoxide anion; HO·, hydroxyl radical.

1. Introduction

In concert with prolongation of the average life span, the accompanying cerebrovascular disorders including dementia have become major social problems in Japan, and effective therapies have been awaited. Chotosan (Diao-Teng-San) is a traditional Chinese/Japanese (Kampo) formula (Table 1), the source of which is *honjiho* (Ben-Shi-Fang) first documented by Xu Shu-Wei (1079-1154). Sohaku

Table 1. Crude drugs composing chotosan

Latin name	Japanese name	Botanical name	Ratio (g)[#]
Uncariae Uncis Cum Ramulus	Chotoko	*Uncaria sinensis* Haviland	3.0
Aurantii Nobilis Pericarpium	Chinpi	*Citrus unshiu* Markovich	3.0
Pinelliae Tuber	Hange	*Pinellia ternata* Breitenbach	3.0
Ophiopogonis Tuber	Bakumondo	*Ophiopogon japonicus* Ker-Gawler	3.0
Poria	Bukuryo	*Poria cocos* Wolf	3.0
Ginseng Radix	Ninjin	*Panax ginseng* C. A. Meyer	3.0
Chrysanthemi Flos	Kikka	*Chrysanthemum morifolium* Ramatulle	3.0
Saposhnikoviae Radix	Bofu	*Saposhnikovia divaricata* Schischkin	3.0
Glycyrrhizae Radix	Kanzo	*Glycyrrhiza uralensis* Fisher	1.0
Zingiberis Rhizoma	Shokyo	*Zingiber officinale* Roscoe	1.0
Gypsum Fibrosum	Sekko	$CaSO_4 \cdot 2H_2O$	5.0

[#] Ratio (g) in accordance with the coded prescription used at Toyama University Hospital

*To whom correspondence should be addressed. e-mail : shimada@med.u-toyama.ac.jp

Asada (1814-1894) described in *futsugoyakushitsuhokankuketsu* that this formula cures patients having Ki (Qi) rising like hot flashes, headache, dizziness, shoulder stiffness, and depressive psychosis. These symptoms are interpreted as those coexisting with hypertension or cerebrovascular disorders. Indeed, the efficacy of chotosan as a Kampo extract for ethical use is described as "for chronic headache in middle-aged or older, or hypertensive patient".

We demonstrated before, by double-blind, randomized controlled trial, that chotosan is effective for the improvement of symptoms accompanying vascular dementia. After that, we investigated the pharmacological actions of chotosan by clinical and experimental studies. In this article, we review the clinical and pharmacological effects of chotosan on the basis of our previous studies.

2. Effects of chotosan on vascular dementia

For the purpose of objectively evaluating the efficacy of chotosan on vascular dementia, we set out to perform a multi-center, well-controlled, chotosan versus placebo trial.[1-3] Furthermore, to evaluate its efficacy using more objective criteria, we carried out a multi-center, double-blind randomized controlled trial.[2-4]

2.1. Well-controlled trial

Prior to the double-blind placebo controlled trial, as a preliminary study, we carried out a multi-center, well-controlled, chotosan versus placebo trial.[1]

Patients diagnosed with vascular dementia were selected according to the following criteria: 1) Patients were defined according to the criteria of DSM-III-R,[5] their Carlo Loeb modified ischemic scores[6] were 5 points or more, and their general condition was stable; 2) One month or more had passed since the last stroke such as cerebral infarction, cerebral bleeding, subarachnoidal bleeding, *etc.*; 3) Patients with dementia of Alzheimer type, severe dementia, complicated by other severe diseases, or judged to be inappropriate for this study by the investigators, were excluded from entry into the trial.

Fig. 1 shows the study protocol. The study was designed as a multi-center, placebo-well-controlled, inter-patient trial. Patients were randomly selected to be administered either chotosan extract (TJ-47, Tsumura & Co., 7.5 g/day) or matched placebo after meals three times a day for 12 weeks. The placebo used in this study was made by Tsumura & Co., and could not be distinguished from the active drugs in form, color and taste by a number of examiners before the trial. During the trial, no other major new medication was allowed.

Patients' characteristics were assessed and brain computed tomography or magnetic resonance imaging was done before entry. Global severity rating, global severity ratings of subjective symptoms, neurological symptoms, psychiatric symptoms and disturbance in daily living activities, as well as the severity rating of each symptom were evaluated by the investigators at the beginning, and after 4, 8, 12 weeks of medication by means of a 5-point rating scale (0 = no symptom, 1 = very slightly affected, 2 = slightly affected, 3 = moderately affected, 4 = severely affected). Table 2 describes the respective symptoms evaluated. Global improvement rating, global improvement ratings of subjective symptoms, neurological symptoms, psychiatric symptoms and disturbance in daily living activities, as well as the

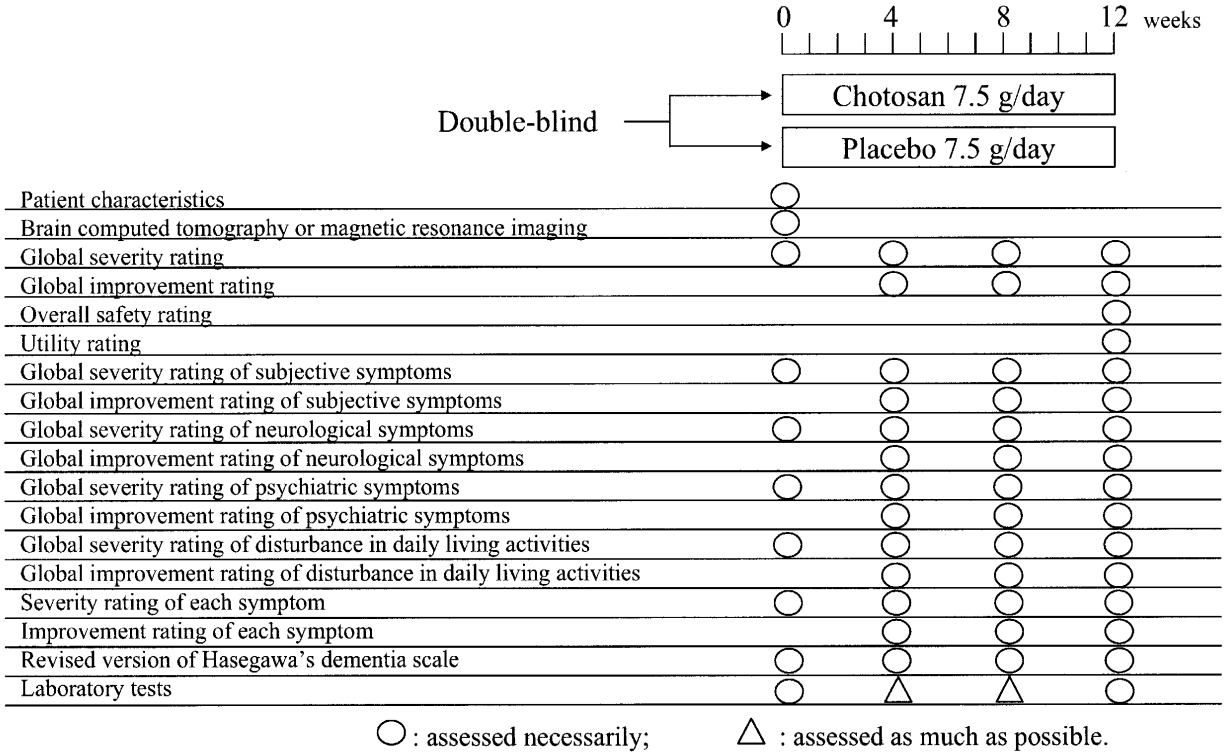

Fig. 1. Study protocol.

Table 2. Symptoms evaluated in the study

Subjective symptoms
Heaviness of head
Headache
Dizziness or vertigo
Shoulder stiffness
Palpitation
Distress feeling of chest
Feeling of hot flushes
Tinnitus
Numbness of limbs
Coldness of limbs
General malaise
Appetite loss
Neurological symptoms
Aphasia
Dysarthria
Motor disturbance
Tremor
Rigidity
Sensory disturbance
Urinary incontinence
Psychiatric symptoms
Spontaneity
Expression of intentions
Interest in television or books
Interest in housework or rehabilitation
Conversation
Global spontaneity
Emotion
Lack of facial expression
Bad humor
Depressed mood
Emotional incontinence
Anxiety
Global emotion
Intellectual ability
Disorientation
Disturbance in short-term memory
Disturbance in long-term memory
Decline in simple arithmetic ability
Global intellectual ability
Abnormal behavior
Hyperkinesia or wandering
Restless or excitement
Nocturnal delirium
Global abnormal behavior
Sleep disturbance
Hallucination or delusion
Disturbance in daily living activities
Sitting
Standing
Walking
Washing face and hands
Putting on and taking off clothes
Having meals regularly
Excretion
Bathing

improvement rating of each symptom were evaluated after 4, 8 and 12 weeks of medication by means of a 6-point rating scale (I = remarkable improvement, II = moderate improvement, III = slight improvement, IV = unchanged, V = aggravation, VI = no symptom both at the beginning and at the point of evaluation). Furthermore, the overall safety and utility ratings were also evaluated at the end of the study. Revised version of Hasegawa's dementia scale (HDS-R)[7] was assessed by the investigators at the beginning, and after 4, 8, 12 weeks of medication. In addition, routine laboratory tests were performed at the beginning and end of the study.

Total enrollment consisted of 60 patients, 9 males and 51 females, and mean age ($\pm$ S.D.) was 78.9 $\pm$ 7.6. The chotosan group consisted of 32 cases and the placebo group 28 cases. There was no statistical difference between the chotosan and placebo groups in terms of sex, age, duration of dementia, causal disease of dementia, complications, rehabilitation and concomitant drugs.

Subjective symptoms. Chotosan was superior to placebo in global improvement ratings of subjective symptoms for each evaluation point at 4 weeks ($p < 0.05$), 8 weeks ($p < 0.01$) and 12 weeks ($p < 0.01$) (Table 3).

Neurological symptoms. There was no statistically significant difference between the chotosan and placebo groups in the global improvement rating of neurological symptoms at any of the evaluation points (Table 3).

Psychiatric symptoms. Chotosan was superior to

Table 3. Global improvement rating in a well-controlled trial

	4 weeks	8 weeks	12 weeks
Patient numbers at each evaluation point			
Chotosan	31	31	31
Placebo	26	26	26
Global improvement rating of subjective symptoms			
Chotosan	0:1:12:14:3	0:3:14:10:2	0:5:11:9:4
Placebo	0:0:4:16:4	0:0:5:11:8	0:0:5:9:10
	$p < 0.05$	$p < 0.01$	$p < 0.01$
Global improvement rating of neurological symptoms			
Chotosan	0:0:0:20:0	0:0:1:18:1	0:0:3:16:2
Placebo	0:0:1:15:0	0:0:2:14:0	0:0:4:12:0
	N.S.	N.S.	N.S.
Global improvement rating of psychiatric symptoms			
Chotosan	0:3:10:17:1	0:1:17:10:3	0:2:16:10:3
Placebo	0:0:4:17:4	0:0:3:15:7	0:1:5:10:9
	$p < 0.05$	$p < 0.01$	$p < 0.01$
Global improvement rating of disturbance in daily living activities			
Chotosan	0:0:4:19:1	0:1:6:15:2	0:1:8:14:1
Placebo	0:0:0:14:3	0:0:2:11:4	0:0:3:8:5
	$p < 0.05$	N.S.	$p < 0.05$
Global improvement rating			
Chotosan	0:2:9:20:0	0:4:15:11:1	0:8:14:8:1
Placebo	0:0:4:19:3	0:0:5:18:3	0:2:4:15:5
	$p < 0.05$	$p < 0.01$	$p < 0.01$

Values of chotosan and placebo represent the number of patients evaluated as remarkable improvement : moderate improvement : slight improvement: unchanged : aggravation, respectively. Others were evaluated as no symptoms. p values by Mann-Whitney test. N.S., not significant.

Table 4. Utility rating in a well-controlled trial

	Very useful	Useful	Slightly useful	Useless	Harmful
Chotosan	2	12	11	6	1
Placebo	0	2	5	17	4

$p < 0.01$ by Mann-Whitney test.

Table 5. Global improvement rating in a double-blind trial

	4 weeks	8 weeks	12 weeks
Patient numbers at each evaluation point			
Chotosan	56	55	55
Placebo	64	63	64
Global improvement rating of subjective symptoms			
Chotosan	1:6:21:17:5	3:10:21:9:5	3:14:20:7:3
Placebo	0:4:17:25:7	2:4:20:15:12	3:4:25:8:14
	N.S.	$p < 0.05$	$p < 0.01$
Global improvement rating of neurological symptoms			
Chotosan	1:1:4:32:3	1:1:7:30:2	1:1:11:26:3
Placebo	0:0:6:41:1	0:1:9:35:1	2:0:9:34:2
	N.S.	N.S.	N.S.
Global improvement rating of psychiatric symptoms			
Chotosan	1:2:23:25:5	2:8:28:14:3	4:14:19:13:5
Placebo	1:0:16:36:11	1:2:19:22:19	1:4:22:19:18
	$p < 0.05$	$p < 0.001$	$p < 0.001$
Global improvement rating of disturbance in daily living activities			
Chotosan	1:1:7:31:3	1:2:12:25:2	1:5:10:24:2
Placebo	0:0:7:35:4	0:0:11:30:5	0:1:11:25:9
	N.S.	N.S.	$p < 0.05$
Global improvement rating			
Chotosan	1:4:22:26:3	3:12:24:13:3	7:14:25:5:4
Placebo	0:1:21:40:2	1:5:21:29:7	1:7:23:21:12
	N.S.	$p < 0.01$	$p < 0.001$

Values of chotosan and placebo represent the number of patients evaluated as remarkable improvement : moderate improvement : slight improvement: unchanged : aggravation, respectively. Others were evaluated as no symptoms. p values by Mann-Whitney test. N.S., not significant.

Table 6. Utility rating in a double-blind trial

	Very useful	Useful	Slightly useful	Useless	Harmful
Chotosan	8	18	18	13	2
Placebo	1	12	20	25	8

$p < 0.01$ by Mann-Whitney test.

placebo in the global improvement rating of psychiatric symptoms at 4 weeks ($p < 0.05$), 8 weeks ($p < 0.01$) and 12 weeks ($p < 0.01$) (Table 3).

Disturbance in daily living activities. Chotosan was superior to placebo in the global improvement rating of disturbance in daily living activities at the evaluation points of 4 weeks ($p < 0.05$) and 12 weeks ($p < 0.05$) (Table 3).

HDS-R. Between mean HDS-Rs of the chotosan and placebo groups, there was no statistical significance at any of the evaluation points of 4, 8 or 12 weeks.

Global improvement rating. Chotosan was superior to placebo in the global improvement rating for each evaluation point at 4 weeks ($p < 0.05$), 8 weeks ($p < 0.01$) and 12 weeks ($p < 0.01$) (Table 3).

Utility rating. Chotosan was superior to placebo in the utility rating ($p < 0.01$) (Table 4).

2.2. Double-blind randomized controlled trial

Following the well-controlled trial, we carried out a multi-center, double-blind randomized placebo-controlled trial.[4)]

Criteria of patient selection and methods were essentially the same as in the previous well-controlled study,[1)] except for double-blind randomization.

Total enrollment consisted of 139 patients, 50 males and 89 females, and mean age (± S.D.) was 76.6 ± 8.4. The chotosan and placebo groups consisted of 69 cases and 70 cases, respectively. There was no statistical difference between the chotosan and placebo groups in terms of sex, age, duration of dementia, causal disease of dementia, complications, rehabilitation and concomitant drugs.

Subjective symptoms. Chotosan was superior to placebo in the global improvement rating of subjective symptoms for evaluation points at 8 weeks ($p < 0.05$) and 12 weeks ($p < 0.01$) (Table 5).

Neurological symptoms. There was no statistical significance between the chotosan and placebo groups in the global improvement rating of neurological symptoms at any of the evaluation points (Table 5).

Psychiatric symptoms. Chotosan was superior to placebo in the global improvement rating of psychiatric symptoms at 4 weeks ($p < 0.05$), 8 weeks ($p < 0.001$), and 12 weeks ($p < 0.001$) (Table 5). Chotosan showed superiority to placebo in the improvement rating of such psychiatric symptoms as spontaneity of conversation at 8 weeks ($p < 0.05$), lack of facial expression at 8 weeks ($p < 0.05$), decline in simple arithmetic ability at 12 weeks ($p < 0.01$), nocturnal delirium at 8 weeks ($p < 0.05$), sleep disturbance at 8 weeks ($p < 0.05$) and 12 weeks ($p < 0.001$), and hallucination or delusion at 8 weeks ($p < 0.05$) and 12 weeks ($p < 0.001$).

Disturbance in daily living activities. Chotosan was superior to placebo in the global improvement rating of disturbance in daily living activities at 12 weeks ($p < 0.05$) (Table 5).

HDS-R. There was no statistical significance between the chotosan and placebo groups in the changes of HDS-Rs from the beginning point (chotosan, 15.3 ± 4.5; placebo, 15.1 ± 4.4) at the evaluation points of 4 weeks (16.7 ± 5.9; 16.6 ± 4.9), 8 weeks (18.0 ± 6.4; 17.3 ± 5.3) and 12 weeks (19.3 ± 6.6; 17.4 ± 6.0). The average HDS-R in the chotosan group tended to be higher than that in the placebo group at 12 weeks, but without statistical significance ($p < 0.1$)

Global improvement rating. Chotosan was superior to placebo in global improvement rating at 8 weeks ($p < 0.01$) and 12 weeks ($p < 0.001$) (Table 5).

Overall safety rating. There was no statistically significant difference between the chotosan and placebo groups in

terms of overall safety rating. It was reported that 5 chotosan and 2 placebo cases possibly suffered adverse effects: urticaria, diarrhea, appetite loss, heartburn and hypertension in the chotosan group, and oral bitterness and liver dysfunction in the placebo group. In all these cases, their complaints disappeared during the course of the trial or after drug discontinuation.

Utility rating. Chotosan showed superiority to placebo in the utility rating ($p < 0.001$) (Table 6).

3. Effects of chotosan on asymptomatic cerebral infarction

On the basis of the efficacy of chotosan on vascular dementia,[1,4] we investigated the effects of chotosan on microcirculation, hemoreological factors, and other related parameters in patients with asymptomatic cerebral infarction.[8,9]

3.1. Microcirculation and hemorheological factors

At first, we examined the effect of chotosan on the microcirculation of bulbar conjunctiva and hemorheological factors in patients with asymptomatic cerebral infarction.[8]

Sixteen patients, 4 males and 12 females, mean age 63.5 ± 8.1 (S.D.) years, with asymptomatic cerebral infarction were evaluated. Their diagnosis was reached by magnetic resonance imaging. Although some of them were being treated by Western medicines that influenced hemorheological factors, such medicines had not been changed from 3 months prior to, and during the chotosan administration.

Chotosan used in this study was prepared as a decoction. The constitution of chotosan is shown in Table 1. Patients were given the chotosan decoction orally 3 times a day (300 ml/day) for 4 weeks.

Before and after the 4-week period of chotosan administration, the microcirculation of bulbar conjunctiva was observed by video-microscope system[10] at about 9:00 a.m. after overnight fasting. At the same time, blood was withdrawn from the cubital vein.

Microcirculatory flow. Fig. 2 shows the changes in microcirculatory flow in the bulbar conjunctiva after 4-week chotosan administration. Internal diameter and flow velocity had increased significantly and, as a consequence, flow volume had also increased significantly.

Erythrocyte aggregability. The maximum diameter of a column of intravascular erythrocyte aggregation (DEA) was defined as the maximum diameter of the largest venule of the bulbar conjunctiva in which intravascular erythrocyte aggregation was observed by video-microscope system. We had already shown that DEA served as a useful index for evaluating erythrocyte aggregability *in vivo*.[11] DEA decreased significantly after 4-week chotosan administration.

Erythrocyte deformability. In accordance with our previous report,[12] we evaluated erythrocyte deformability by measuring the filtration time required for 400 μl of 15% red blood cell suspension to pass through a 5-μm pore filter under constant -10 H_2O pressure. The index of erythrocyte deformability, the filtration time, shortened significantly after the administration of chotosan for 4 weeks.

3.2. Serum nitric oxide, lipid peroxidase

A decrease of nitric oxide (NO) causes endothelial dysfunction and as well as blood insufficiency.[13] NO is an extremely unstable molecule and rapidly undergoes oxidative degradation to stable inorganic oxides, nitrite/nitrate (NO_2^-/NO_3^-). Lipid peroxide (LPO) is useful as an index of free radicals in blood. Therefore, we investigated the effects of chotosan on serum NO_2^-/NO_3^- and LPO levels in patients with asymptomatic cerebral infarction.[9]

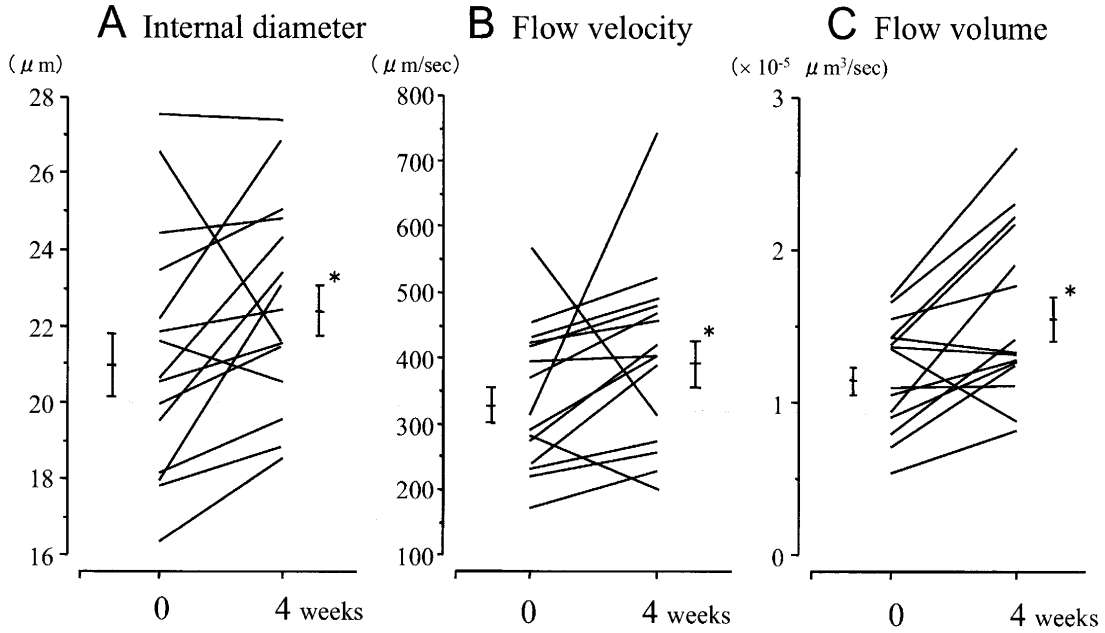

Fig. 2. Changes in internal diameter (A), flow velocity (B) and flow volume (C) in bulbar conjunctiva before and after the oral administration of chotosan for 4 weeks in patients with asymptomatic cerebral infarction. Data are mean ± S.E. (n = 16). *$p < 0.05$.

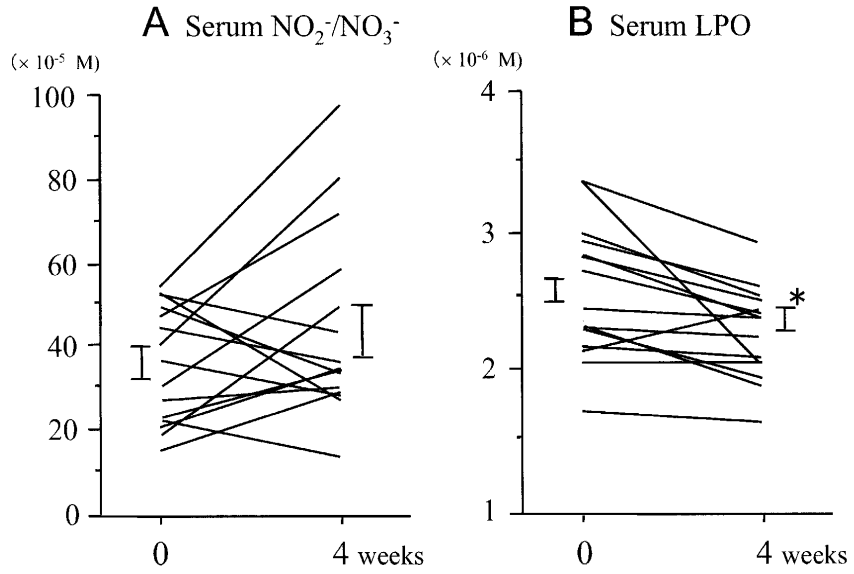

Fig. 3. Changes in serum NO_2^-/NO_3^- (A) and LPO (B) before and after the oral administration of chotosan for 4 weeks in patients with asymptomatic cerebral infarction. Data are mean ± S.E. (n = 15). *$p < 0.05$.

Fifteen patients, 4 males and 11 females, mean (± S.D.) age 63.7 ± 7.8 years, were administered chotosan for 4 weeks.

Serum NO_2^-/NO_3^-: Serum NO_2^-/NO_3^- was measured with an automated system, ECO-10 (EICOM Co., Kyoto), based on the Griess reaction.[14] Fig. 3A shows the changes in serum NO_2^-/NO_3^- after the administration of chotosan for 4 weeks. Serum NO_2^-/NO_3^- tended to increase, but without significance.

Serum LPO: Serum LPO decreased significantly after the administration of chotosan for 4 weeks (Fig. 3B).

4. Effects of chotosan on stroke-prone spontaneously hypertensive rats

We conducted experimental studies on the basis of clinical results. First, we investigated the effects of chotosan on stroke-prone spontaneously hypertensive rats (SHR-SP).[15,16]

4.1. Effects of chotosan on hemorheological factors and vascular function in SHR-SP

We investigated whether chotosan improved hemorheological factors and vascular function on SHR-SP.[15] Sixteen 7-week-old male SHR-SP and chotosan extract donated by Tsumura & Co. were used in the study. Rats were randomly assigned to 2 groups. The control group received distilled water, and the chotosan group received 450 mg/kg/day of chotosan extract in drinking water for 8 weeks.

Blood pressure, serum NO_2^-/NO_3^-, LPO, and erythrocyte deformability. Table 7 shows the results of blood pressure, serum NO_2^-/NO_3^-, LPO and erythrocyte deformability. Blood pressure was measured by the tail-cuff method. Systolic and mean blood pressure of the chotosan group significantly decreased as compared to the control group. In the chotosan group, serum NO_2^-/NO_3^- levels tended to increase compared to the control, but without significance. Serum LPO significantly decreased compared to the control. Erythrocyte deformability improved significantly in comparison to the control group.

Vascular function. Three-mm aortic ring preparations from rats were mounted on steel hooks in a Magnus chamber, and relaxation experiments were performed. One end of the aorta was attached to a force-displacement transducer so that its isometric contraction could be recorded. The rings were precontracted with 5×10^{-7} M norepinephrine (NE).

Table 7. Blood pressure, serum NO_2^-/NO_3^-, LPO and erythrocyte deformability in SHR-SP

Group	0 weeks		8 weeks	
	Control	Chotosan	Control	Chotosan
Systolic blood pressure (mm Hg)	152.5 ± 6.2	154.6 ± 4.8	243.1 ± 4.7	214.9 ± 4.7*
Mean blood pressure (mm Hg)	128.2 ± 5.6	126.4 ± 7.2	202.5 ± 6.6	179.6 ± 7.2*
Serum NO_2^-/NO_3^- ($\times 10^{-5}$ M)			16.5 ± 0.9	17.9 ± 2.1
Serum LPO ($\times 10^{-6}$ M)			2.36 ± 0.07	2.23 ± 0.05*
Erythrocyte deformability (msec)			6.16 ± 0.29	5.28 ± 0.14**

Each value is mean ± S.E. (n = 8). *$p < 0.05$, **$p < 0.01$ compared to control.

For endothelium-dependent relaxations, vessels were relaxed with acetylcholine (Ach). To study the direct relaxation of vascular smooth muscle, vessels were relaxed with sodium nitroprusside (SNP). Relaxation was expressed as percentage of decrease in maximal tension obtained by NE-induced contraction.

Ach induced endothelium-dependent relaxation, reaching a maximum at 10^{-4} M, and relaxation of aortas in the chotosan group increased to a greater degree than in the control group, significantly (Fig. 4A). In endothelium-independent relaxation induced by SNP, there was no significant difference between the two groups (Fig. 4B).

4.2. Effects of chotosan on stroke and life span in SHR-SP

Next, we tried to investigate whether chotosan prevents the occurrence of stroke and prolongs life span in SHR-SP.[16]

Twenty-four 8-week-old male SHR-SPs were used. Chotosan extract used in the study was prepared in our laboratory by extracting the mixture of 11 kinds of crude drugs (Table 1) with boiling water and converting to freeze-dried powder. The SHR-SPs were randomized into three groups. Two groups were given either 0.1% (150 mg/kg/day) or 0.3% (450 mg/kg/day) of chotosan extract dissolved in drinking water from 8 weeks of age. The third group (control) was given water not containing the extract. All animals were inspected daily for neurological and behavioral signs accompanying stroke, *i.e.* paralysis, convulsion, involuntary movement, jumping, aggressivity and prostration, until death.

Occurrence of stroke. The mean age at which any of the neurological and behavioral signs first appeared was 102.8 ± 5.3 (S.E.) days in the control group, 122.6 ± 10.8 days in the 0.1% chotosan group and 159.6 ± 19.1 days in the 0.3% chotosan group; that of the 0.3% group was significantly longer compared to the control group. The cumulative percent occurrence of neurological and behavioral signs in the 0.3% chotosan group was significantly inhibited compared to control (Kaplan-Meier analysis followed by log-rank test).

Survival. Fig. 5 shows the survival of the three groups. The mean survival times of the control group, 0.1% and 0.3% chotosan group were 122.1 ± 7.9, 159.8 ± 6.1 and 176.8 ± 22.7 days, respectively. The survival time of the 0.3% chotosan group was significantly longer than that of the control group. The percent survivals of both the 0.1% and 0.3% chotosan groups were significantly enhanced compared to control.

5. Effects of *Uncaria sinensis* on vascular function

Uncaria sinensis Haviland (US) is recognized as the most important of the crude drugs comprising chotosan. According to the results of the effects of chotosan on vascular function, we focused our studies on US.[17,18]

5.1. Vasodilator effects of *Uncaria sinensis*

We studied the vasodilator effects of US and its phenolic and alkaloid fractions *in vitro*.[17]

The extract was obtained by boiling US in water for 2 h and then freeze-drying to a resultant powder (*Uncaria sinensis* extract (USE)). We obtained 38.2 g of extract from 320 g of raw material. USE was dissolved in water and then subjected to Diaon HP-20 chromatography, and successively eluted with water, 50% aqueous MeOH, and finally MeOH. Each eluate was lyophilized to afford a water fraction (19.43 g; water eluate (USE-W)), a 50% aqueous methanol fraction (9.60 g, phenolic fraction (USE-P)), and a methanol fraction (0.53 g; alkaloid fraction (USE-A)),

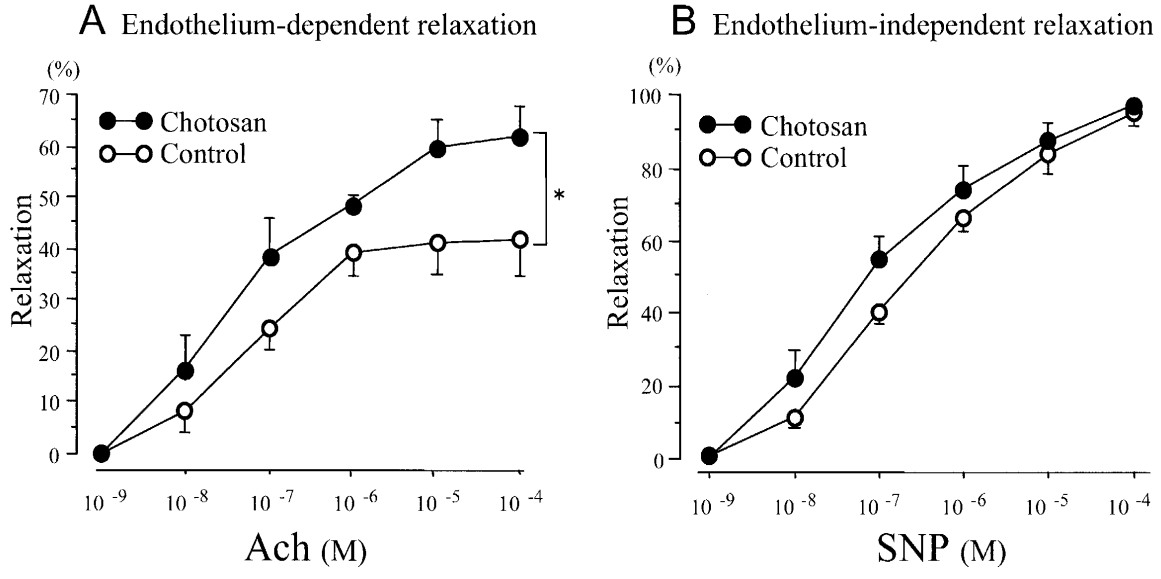

Fig. 4. Endothelium-dependent relaxation induced by Ach (A) and endothelium-independent relaxation induced by SNP (B) in aorta of SHR-SP. Values are expressed as percentage of decrease in maximal tension contracted with 5×10^{-7} M NA. Data are mean ± S.E. (n = 8). *$p < 0.05$.

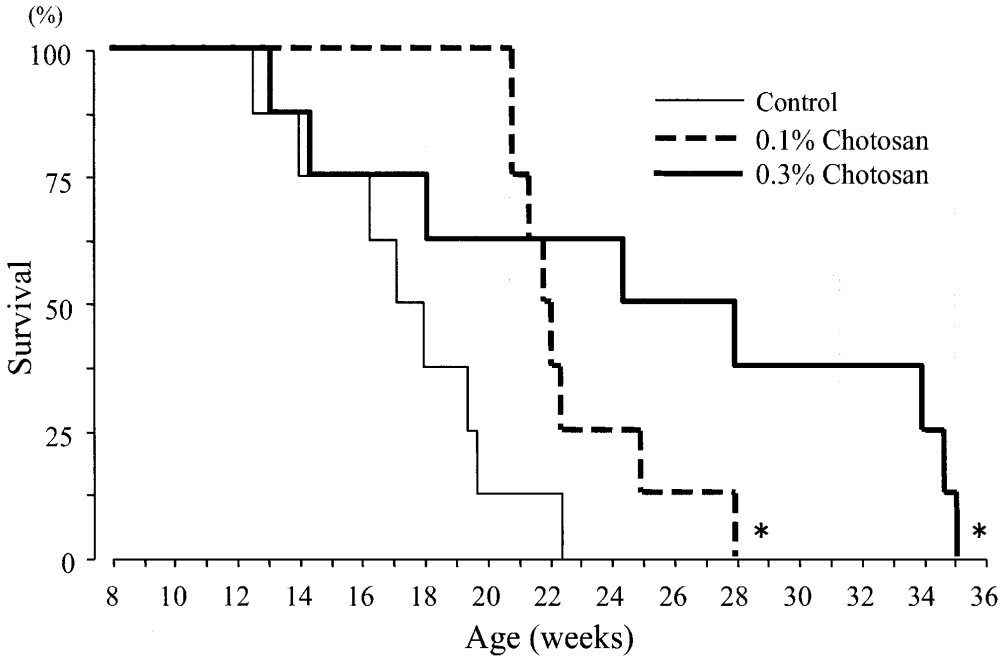

Fig. 5. Percent survival of 0.1% chotosan, 0.3% chotosan and control groups. *$p < 0.05$ compared to control.

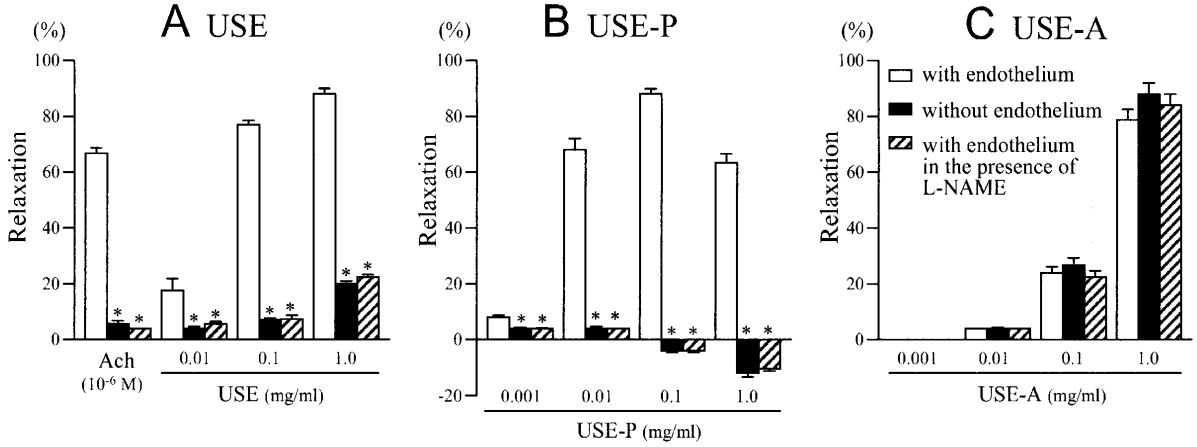

Fig. 6. Dose-response relationship of (USE) (A), USE-P (B) and USE-A (C) -induced relaxation on the $PGF_{2\alpha}$-precontracted aorta. Data are mean ± S.E. (n = 6). *$p < 0.05$ compared to rings with endothelium.

respectively.

Relaxation effects. Aortic strips of male Wistar rats were contracted by treatment with 3×10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in Magnus chambers. When the $PGF_{2\alpha}$-induced contraction reached a plateau, extract was added at concentrations ranging from 1×10^{-6} to 1×10^{-3} g/ml, cumulatively. Relaxation was expressed as percentage of the decrease in maximal tension obtained by $PGF_{2\alpha}$-induded contraction. To investigate the effect of NO, preparations with the endothelium were exposed to 10^{-4} M N^G-nitro-l-arginine methylester (L-NAME), an inhibitor of NO synthesis, for 60 min before precontraction.

Rat aorta with intact endothelium exhibited relaxation with addition of USE dose-dependently. In contrast, relaxation of the rings without endothelium and the rings with endothelium in the presence of L-NAME decreased compared to that with intact endothelium (Fig. 6A). USE-P relaxed the rat aorta with intact endothelium, whereas the rings without endothelium and those in the presence of L-NAME showed no relaxation (Fig. 6B). USE-A relaxed the aorta with intact endothelium. The rings without endothelium and those with endothelium in the presence of L-NAME also showed relaxation to the same degree as seen in aorta with intact endothelium (Fig. 6C). These results indicate that the phenolic fraction of US has endothelial-dependent relaxation effect, and its alkaloid fraction has endothelial-independent relaxation effect.

Inhibitory effects on contraction. First, we investigated the inhibitory effects of USE and its fractions on contraction induced by oxygen-derived free radicals. Contraction was expressed as percentage of the increase in maximal tension obtained by $PGF_{2\alpha}$-induced contraction. During contractions

induced by $PGF_{2\alpha}$, xanthine oxidase (1-9 mU/ml) in the presence of xanthine (10^{-4} M) caused contraction dose-dependently. The contractions in the presence of USE, USE-P, USE-A or superoxide dismutase (SOD) significantly decreased compared to control, indicating that US and its phenolic and alkaloid fractions have inhibitory effects on contraction induced by oxygen-derived free radicals. Next, we examined the inhibitory effects of alkaloid fraction on Ca^{2+}-induced contractions. Changes in the contractile tension developed by USE-A were expressed as percentage of the maximum tension obtained with the $CaCl_2$ addition. Calcium-induced contraction was significantly inhibited by USE-A as well as by verapamil, one of the calcium channel blockers.

5.2. Effects of *Uncaria sinensis* on vasodilation in SHR

Next, we investigated the effects of the oral administration of US on vasodilation in spontaneously hypertensive rats (SHR).[18]

Eight-week-old SHRs and USE prepared in our laboratory were used. Twenty-seven SHRs were assigned to three groups: control group, low US group (150 mg/kg/day), and high US group (450 mg/kg/day). USE was dissolved in water and given to rats for 8 weeks.

Relaxation effects. The rats were sacrificed and their abdominal aorta was used for the experiment. Aortic rings were precontracted with 3×10^{-6} M $PGF_{2\alpha}$. For endothelium-dependent relaxation, vessels were relaxed with Ach (10^{-9} - 10^{-4} mol/L). To study the direct relaxation of vascular smooth muscle, vessels were relaxed with SNP (10^{-9} - 10^{-4} mol/L). Relaxation was expressed as percentage of the decrease in maximal tension obtained by $PGF_{2\alpha}$-induced contraction. Ach-induced endothelium-dependent relaxation of the high US group increased significantly compared to the control group. In SNP-induced endothelium-independent relaxation, there was no significant difference among the three groups.

Inhibitory effects on contraction. Contraction was expressed as percentage of the increase in maximal tension obtained by PGF2 -induced contraction. In vessels with intact endothelium, contraction of the high US group was significantly decreased compared to the control group. Removal of the endothelium decreased contractions, but there were no significant differences among the three groups. Together with the results of the relaxation experiment, we can suggest that US has a protective capability for endothelial function *in vivo*.

6. Neuroprotective effects of *Uncaria sinensis in vitro*

Glutamate is a physiological excitatory transmitter in the central nervous system. However, overactivation of glutamate receptors has been suggested to be involved in several neurological disorders including ischemia-hypoxic injury.[19] NO also plays an important role in the central nervous system, but it is concerned with a final common pathway of a wide variety of neurological disorders including ischemia.[20] Therefore, we investigated the protective effects of US on glutamate- and NO donor-induced neuronal death using cultured cerebellar granule cells.[21-24]

6.1. Effect of *Uncaria sinensis* on glutamate-induced neuronal death

First, we investigated whether US had a protective effect on glutamate-induced neuronal death.[21]

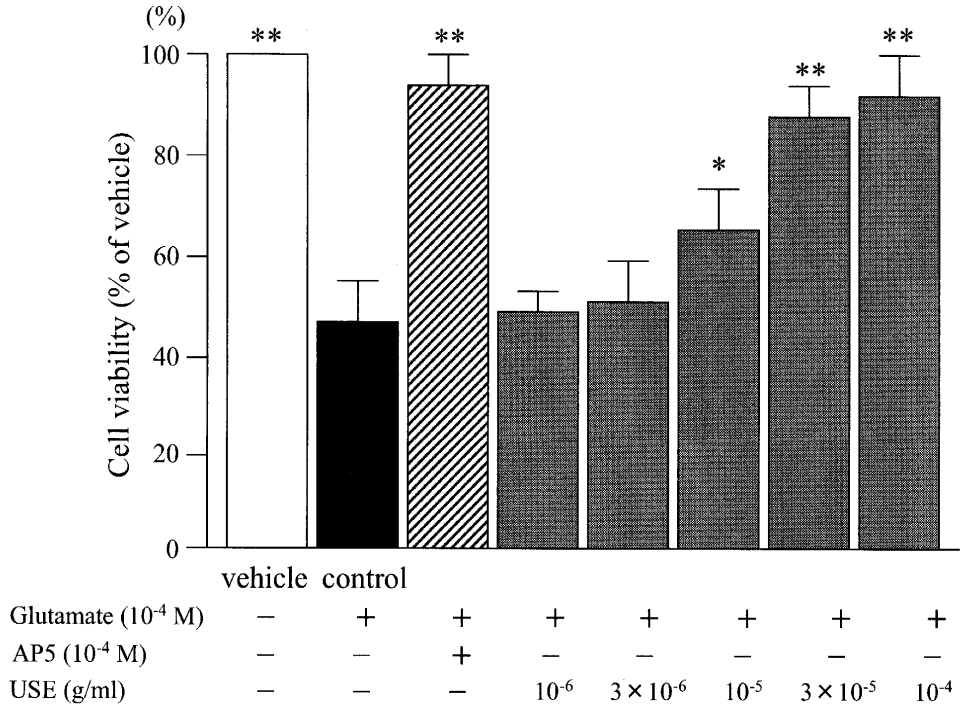

Fig. 7. Effect of USE on cell viability on glutamate-induced neuronal death assessed by MTT assay in cerebellar granule cells. Data are mean ± S.D. (n = 4). *p < 0.05). **p < 0.01 compared to control.

Cerebellar granule cells from 7-8-day-old Wistar rats were cultured, and used at 7-8-days for the experiments. USE was prepared in our laboratory (yield, 7.7%). Cell viability was assessed by MTT assay. Cultured cells were washed 3 times with Mg^{2+}-free Locke's solution, then incubated with Mg^{2+}-free Locke's solution with (control) or without (vehicle) 10^{-4} M glutamate. AP5 (10^{-4} M), a specific *N*-methyl-D-aspartate (NMDA) receptor antagonist, and various concentrations of USE (10^{-6} - 10^{-4} g/ml) were dissolved in Mg^{2+}-free Locke's solution together with glutamate (10^{-4} M), and then applied to cells. After 1 h incubation at 37℃, MTT (5×10^{-4} g/ml) was applied and incubated for 30 min at 37℃. Cells were then washed and lysed in isopropanol with 0.04 N HCl to dissolve the blue formazan products. Optical density was read at 570 nm with a spectrophotometer and expressed as percentage of the vehicle.

Cell viability. Glutamate-induced neuronal death was completely prevented by AP5. The incubation of cells together with glutamate and USE was found to be protective in a dose-dependent manner, and concentrations of 10^{-5} to 10^{-4} g/ml of USE showed significant protection compared to only glutamate exposure (Fig. 7).

6.2. Effects of alkaloids isolated from *Uncaria sinensis* on glutamate-induced neuronal death

Following the result of the protective effect of US on glutamate-induced neuronal death in cultured cerebellar granule cells, we confirmed that USE-A possessed a protective effect on glutamate-induced neuronal death (unpublished observation). Consequently, we evaluated the protective effects of alkaloid-isolated US.[22]

The oxyindole alkaloids corynoxeine, rhynchophylline, isorhynchophylline and isocorynoxeine, and the indole alkaloids geissoschizine methyl ether, hirsuteine and hirsutine were isolated from the hooks and stems of US at Tsumura Central Research Laboratories, as described elsewhere.[25] Cerebellar granule cells from 7-8-day-old Wistar rats were cultured, and used at 7-8-days *in vitro* for the experiments. The basic technique was the same as in our previous study.[21] As alkaloids of US were insoluble in water, they were dissolved in dimethylsulphoxide (DMSO) at a concentration of 10^{-1} M, and all incubation solutions were adjusted to contain 1% DMSO.

Cell viability. Corynoxeine had no significant protective effect on glutamate (10^{-4} M)-induced neuronal death at any concentration (10^{-5} - 10^{-3} M). Rhynchophylline significantly inhibited glutamate-induced cell death at 10^{-3} M. Isorhynchophylline had a distinct dose-dependent protective effect against glutamate-induced cell death, and from 10^{-4} to 10^{-3} M afforded significant protection compared with exposure to glutamate only. Isocorynoxeine from 10^{-4} to 10^{-3} M also significantly inhibited cell death, but the most effective dose was 3×10^{-4} M. Geissoschizine methyl ether had no significant protective effect at any concentration. Hirsuteine and hirsutine from 10^{-4} to 3×10^{-4} M significantly inhibited glutamate-induced cell death, but their effects were weaker than those of isorhynchophylline and isocorynoxeine.

6.3. Effects of phenolic compounds isolated from *Uncaria sinensis* on glutamate-induced neuronal death

Next, we evaluated the protective effects of phenolic compounds isolated from US on glutamate-induced neuronal death.[23] Prior to this study, we confirmed that phenolic fraction possessed a protective effect (unpublished observation).

The phenolic compounds epicatechin, catechin, procyanidin B-1, procyanidin B-2, hyperin and caffeic acid were isolated at Tsumura Central Research Laboratories.[23] The basic technique was the same as in our previous study.[22]

Cell viability. Epicatechin significantly inhibited glutamate (10^{-4} M)-induced neuronal death at concentrations above 10^{-4} M. Catechin had significant protective affect at 3 10^{-4} M. Procyanidin B-1 at from 3×10^{-5} to 3×10^{-4} M significantly inhibited glutamate-induced cell death. Procyanidin B-2 had significant protective effect from 10^{-4} to 3×10^{-4} M. Hyperin and caffeic acid had no significant protective effect at any concentration (3×10^{-6} - 3×10^{-4} M).

6.4. Effects of *Uncaria sinensis* on nitric oxide donor-induced neuronal death

NO is involved in neuronal death induced by ischemia in the central nervous system. We investigated the protective effect of US on NO donor-induced neuronal death in cultured cerebellar granule cells.[24]

USE, USE-W, USE-P and USE-A were prepared in the same manner as in our previous report.[17] Cerebellar granule cells from 8-day-old Wistar rats were cultured, and used at 8-9 days *in vitro* for the experiments. Cell viability was assessed by basically the same technique using MTT assay as in our previous study.[21] Extracts were dissolved in Locke's solution as × 100 concentrated stock solution, and added to the original culture medium. Ten minutes later, the NO donors, SNP and 3-morpholinosydnomimine (SIN-1), were dissolved in Locke's solution as × 100 concentrated solution and added to the culture medium. The cell cultures were incubated with drugs at 37℃ with 5% CO_2 in a humidified incubator for certain periods.

Cell viability. Fig. 8 shows the time course of SNP (3×10^{-5} M) and SIN-1 (3×10^{-4} M)-induced neuronal death and the protective effect of USE as evaluated by MTT assay. Application of SNP or SIN-1 for 3 h did not influence cell viability, but longer applications decreased cell viability. USE (10^{-5}, 3×10^{-5} and 10^{-4} g/ml) significantly prevented SNP-induced cell death at 6, 12 and 24 h. USE (10^{-5} and 3×10^{-5} g/ml) provided significant protection against neuronal death induced by SIN-1 at 6, 12 and 24 h. We also examined the protective effect of USE-W, USE-P and USE-A on SNP- and SIN-1-induced neuronal death. USE-W had no protective ability. USE-P (10^{-5} and 3×10^{-5} g/ml) and USE-A (3×10^{-5} and 10^{-4} g/ml) provided protection on neuronal death induced by SNP (3×10^{-5} M, 24 h) in a dose-dependent manner. USE-P (3×10^{-6} and 10^{-5} g/ml) and USE-A (3×10^{-5} and 10^{-4} g/ml) also had protective effects on neuronal death induced by SIN-1 ($3 \times$

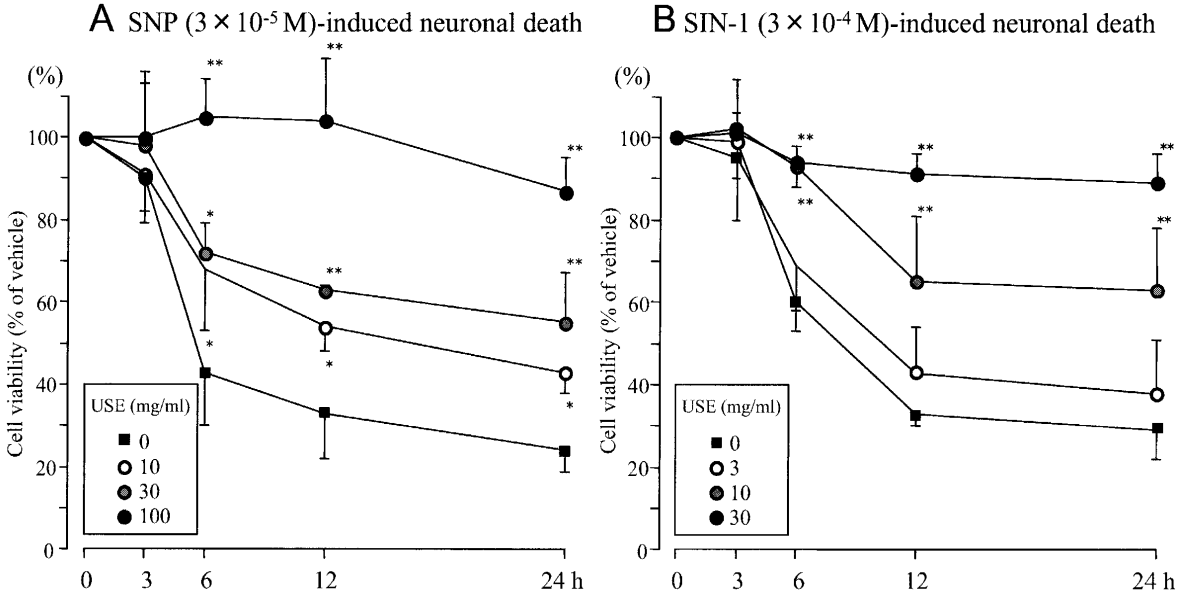

Fig. 8. Effect of USE on cell viability against SNP (3×10-5 M)-induced neuronal death (A) and SIN-1 (3×10^{-4} M)-induced neuronal death (B) assessed by MTT assay in cerebellar granule cells. Data are mean ± S.D. (n = 4). *$p < 0.05$, **$p < 0.01$ compared to control at each time point.

10^{-4} M, 24 h) in a dose-dependent manner. These results indicate that US and its phenolic and alkaloid fractions possess protective ability against NO-induced neuronal death.

7. Neuroprotective effects of chotosan and *Uncaria sinensis in vivo*

Following the studies of the neuroprotective effects of US *in vitro*, we tried to investigate the neuroprotective effects of chotosan and US *in vivo* using a transient forebrain ischemia model of gerbil.[26,27]

7.1. Effects on delayed neuronal death after transient brain ischemia

First, we investigated the protective effects of chotosan and US against delayed neuronal death of the pyramidal hippocampal CA1 region after forebrain ischemia/reperfusion (i/rp), and LPO and NO_2^-/NO_3^- levels in the homogenized hippocampus.[26]

The decoctions of chotosan and US were converted to freeze-dried powder, and used in the study. Adult male Mongolian gerbils (10 weeks old, 60-65 g) were used. Two doses (1.0 and 3.0%) of chotosan extract (CSE) were dissolved in drinking water and the animals were given access ad libitum. The animals were randomly divided into the following 4 groups: sham-operated group (sham), sham operation without CSE-treatment; control group (control), i/rp without CSE-treatment; 1.0% CSE group (1.0% CSE), i/rp with 1.0% CSE-treatment; 3.0% CSE group (3.0% CSE), i/rp with 3.0% CSE-treatment. CSE was administered to animals from 7 days prior to i/rp until 7 days after i/rp. The USE-treatment protocol was essentially the same.

Surgical procedures were performed according to the method of Kirino[28] with slight modification. The carotid arteries were exposed, and they were occluded for exactly 4 min with microaneurysm clips. The clips were then removed and reperfusion was visually confirmed before the neck was closed with silk threads. At 7 days after i/rp, brains were removed, and the numbers of viable cell bodies remaining in three portions of CA1 (medial, intermediate, and lateral) in both the left and right hemispheres of the brain were counted under a microscope at × 400 magnification. Their total number was expressed as percentage of the average of sham-operated animals.

For the preparation of brain homogenate supernatant, hippocampi were quickly separated from freshly removed brain samples and washed in ice-cold PBS (pH 7.4). They were then minced in ice-cold PBS and homogenized at a ratio of 1:10, w:v. After centrifugation of brain homogenates at 3,000 g for 10 min at 4℃, they were used for measurements of LPO and NO_2^-/NO_3^-.

Delayed neuronal death. All groups drank approximately 6 mL/animal/day (100 mL/kg/day) of water, allowing the calculation that the 1.0 and 3.0% CSE and USE groups ingested approximately 1.0 and 3.0 g/kg/day of CSE or USE, respectively. Histological examination revealed that the numbers of viable pyramidal cells in the hippocampal CA1 region at 7 days after i/rp in the 1.0 and 3.0% CSE groups, administered CSE from 7 days prior to i/rp until 7 days after i/rp, were significantly greater than in control (Fig. 9A). Similarly, those in this region at 7 days after i/rp in the 3.0% USE group, following the same administration schedule, were also significantly greater than in control (Fig. 9B).

LPO and NO_2^-/NO_3^-. Both LPO and NO_2^-/NO_3^- levels in the homogenized hippocampus at 48 h after i/rp in both 1.0% CSE and 3.0% USE groups, treated from 7 days prior until 48 r after i/rp, were significantly lower than those of

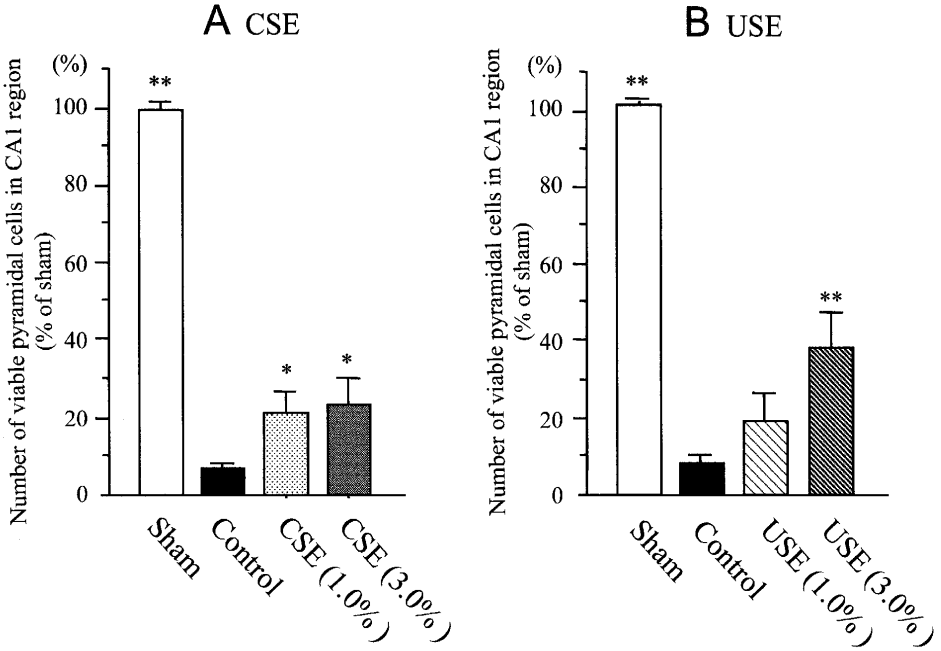

Fig. 9. Neuroprotective effects of CSE (A) and USE (B) on transient forebrain ischemia in gerbils. Data are the number of viable pyramidal cells in hippocampal CA1 region at 7 days after i/rp (mean ± S.E., n = 10). $*p < 0.05$, $**p < 0.01$ compared to control.

control.

7.2. Effects on antioxidant enzyme activities after transient brain ischemia

Next, for the purpose of clarifying whether endogenous antioxidant enzymes contribute to the neuroprotective effects of chotosan and US, we investigated the effects of CSE and USE on SOD, catalase (CAT) and glutathione peroxidase (GSH-Px) activities, and also on superoxide anion ($O_2^{-}\cdot$) and hydroxyl radical (HO·) scavenging activities in the brain, by using the transient forebrain ischemia model of gerbils.[27] The basic experimental technique was the same as in our previous report.[26] Measurement of $O_2^{-}\cdot$ and HO· scavenging activities was performed by electron spin resonance technique.[29]

SOD activity was determined according to the nitrous acid method previously described, which is based on the inhibition of nitrite formation by hydroxylamine in the presence of $O_2^{-}\cdot$ generation.[30,31] CAT activity was evaluated by following the decomposition of H_2O_2 directly by monitoring the decrease in extinction at 240 nm.[32] GSH-Px activity was measured by colorimetric assay that determined the concentration of 2-nitro-5-thiobenzoic acid, a compound produced through the reaction between glutathione and 5,5'-dithiobis-2-nitrobenzoic acid.[33]

Effects of CSE and USE on $O_2^{-}\cdot$ and HO· scavenging activities. The $O_2^{-}\cdot$ and HO· scavenging activities of the homogenized hippocampus and cortex obtained from gerbils without i/rp after 7-day continuous oral administration of 1.0% CSE or 3.0% USE were significantly higher than those of non-treated control.

Effects of CSE and USE on antioxidant enzyme activities. After 7-day continuous oral administration of 1.0% CSE or 3.0% USE without the i/rp procedure, CAT activities were significantly higher than those of non-treated control, but SOD and GSH-Px activities were not affected in either hippocampus or cortex (Fig. 10).

Effects of CSE and USE on $O_2^{-}\cdot$ scavenging activities after i/rp. The $O_2^{-}\cdot$ scavenging activities in the hippocampus of both the CSE and USE groups were significantly higher than those of the control group at 3 h and 7 days after i/rp. In the cortex, the $O_2^{-}\cdot$ scavenging activities of both the CSE and USE groups were significantly higher than those of the control group at all three time points of 3 h, 48 h and 7 days after i/rp.

Effects of CSE and USE on HO· scavenging activities after i/rp. The HO· scavenging activities in the hippocampus of both the CSE and USE groups were significantly higher than those of control at 3 h and 7 days after i/rp. In the cortex, the HO· scavenging activities of the CSE group were significantly higher than those of control at 3 h and 7 days after i/rp, and those of the USE group were significantly higher than those of the control group at all three time points after i/rp.

Effects of CSE and USE on CAT activities after i/rp. The hippocampal CAT activity of the CSE group was significantly higher than that of control at 7 days after i/rp, but lower than control at 48 h after i/rp. The CAT activities in the hippocampus of the USE group were significantly higher than those of control at 3 h and 7 days after i/rp, but lower than control at 48 h after i/rp (Fig. 11A). In the cortex, the CAT activity of the CSE group was significantly higher than that of control at 3 h after i/rp. The CAT activities in the cortex of the USE group were significantly higher

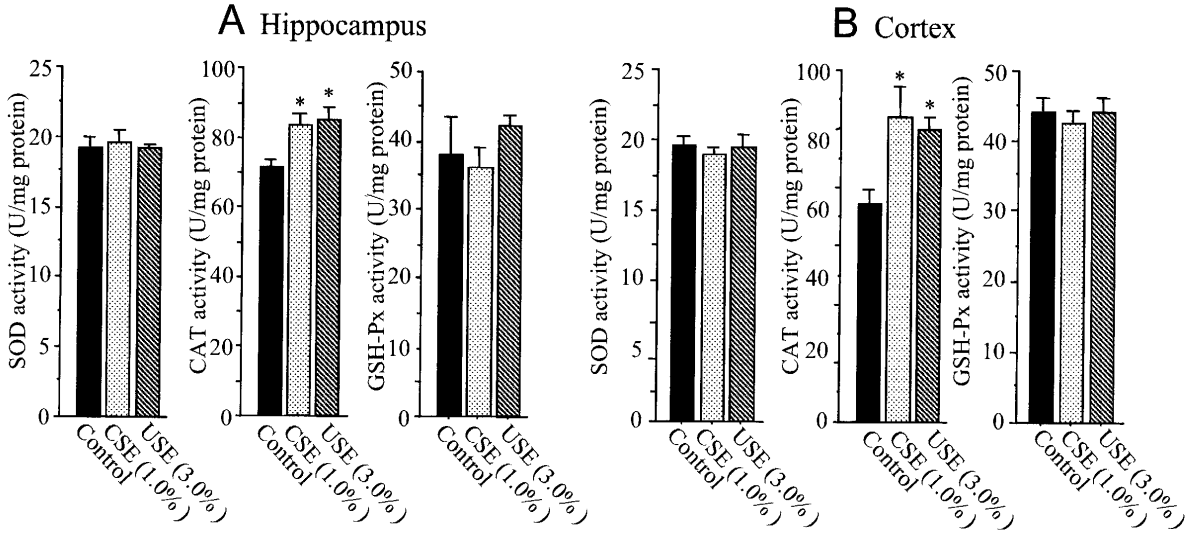

Fig. 10. Effects of 7-day continuous oral administration of 1.0% CSE or 3.0% USE on SOD, CAT and GSH-Px activities in homogenized hippocampus (A) and cortex (B) of gerbils without i/rp. Data are mean ± S.E. (n = 7-8). *$p < 0.05$ compared to control.

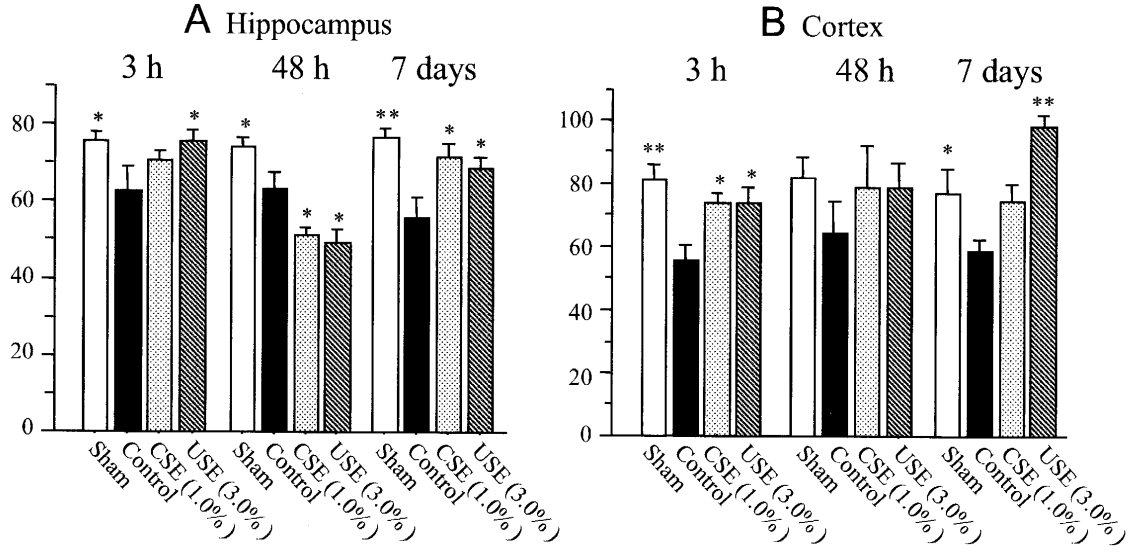

Fig. 11. Effects of oral administration of 1.0% CSE or 3.0% USE on CAT activities in homogenized hippocampus (A) and cortex (B) of gerbils at 3 h, 48 h and 7 days after i/rp. Data are mean ± S.E. (n = 7-8). *$p < 0.05$, **$p < 0.01$ compared to control.

than those of control at 3 h and 7 days after i/rp (Fig. 11B).

8. Conclusion

The effects of chotosan, based on the results of our past studies, are summarized in schematic form in Fig. 12. Chotosan has multiple pharmacological actions such as improving effect on microcirculation, protective effect on endothelial function, and a neuroprotective effect, and this formula is considered to be useful for prevention of the development of cerebrovascular disorders. Other researchers are approaching the clarification of the pharmacological actions of chotosan from different viewpoints. We strongly expect further accumulations of clinical and pharmacological evidence of the activity of this formula.

Acknowledgment

This work was partially supported by a Grant-in-Aid for Scientific Research (C) No. 16590556 from the Japan Society for the Promotion of Science, and a Grant-in-Aid for the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

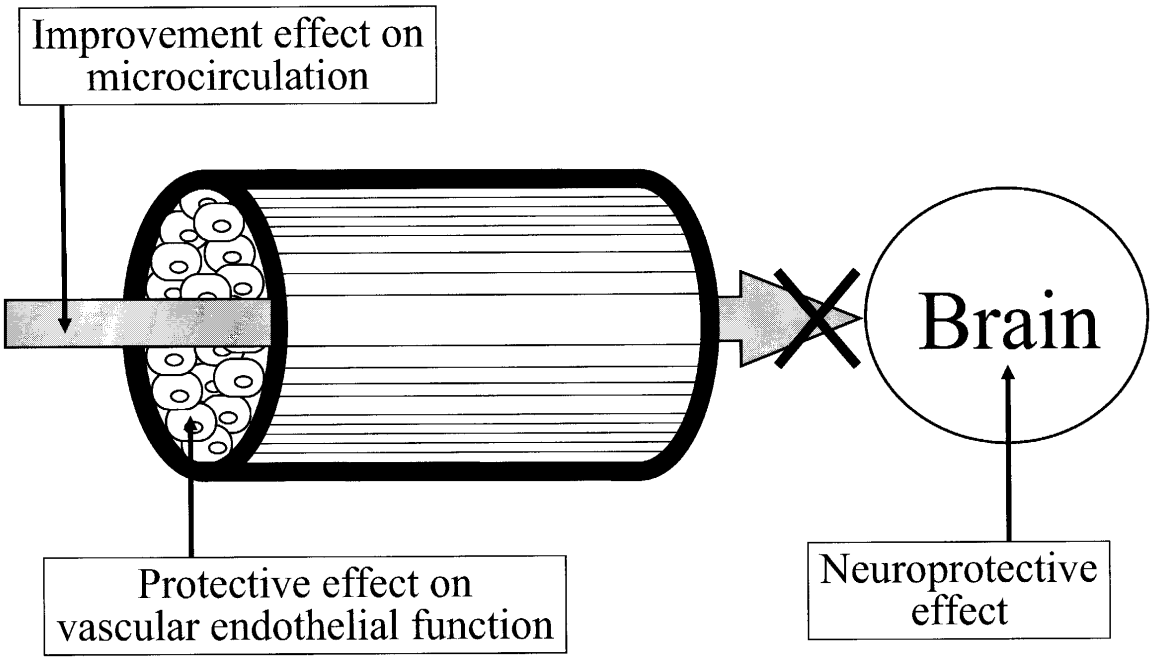

Fig. 12. Pharmacological actions of chotosan.

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Japanese abstract

これまでに我々が行ってきた漢方方剤・釣藤散の脳血管障害に関する臨床及び基礎研究を概説した。二重盲検ランダム化比較臨床試験によって脳血管性認知症に対する釣藤散の有用性が明らかとなった。無症候性脳梗塞患者を対象とした臨床研究では，眼球結膜微小循環と血液レオロジー因子を改善した。脳卒中易発症自然発症高血圧ラットを用いた基礎研究では，血圧上昇抑制作用，血管内皮保護作用，さらには延命作用が明らかとなった。釣藤散の主要構成生薬である釣藤鉤のフェノール画分は内皮依存性血管弛緩作用を，アルカロイド画分は内皮非依存性血管弛緩作用を有していた。培養神経細胞を用いた *in vitro* の実験で，釣藤鉤はグルタミン酸及び一酸化窒素（NO）供与体によって誘導される神経細胞死に対して保護作用を示した。スナネズミを用いた *in vivo* の実験で，釣藤散及び釣藤鉤は一過性脳虚血による海馬体CA1領域の錐体細胞の遅発性神経細胞死を抑制し，脳組織のスーパーオキサイドとヒドロキシルラジカルの消去活性及び抗酸化酵素の中ではカタラーゼ活性を高めた。以上より，釣藤散は微小循環改善作用，血管内皮機能保護作用及び神経保護作用などの多面的な薬理作用を有し，脳血管障害の発症及び進展予防に有用な薬剤であると考えられる。

*〒930-0194 富山市杉谷2630
富山大学医学部和漢診療学講座 嶋田 豊