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Protective effects of Choto-san and hooks and stems of *Uncaria sinensis* against delayed neuronal death after transient forebrain ischemia in gerbil

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Abstract

Previously, we revealed that Choto-san (Diao-teng-san in Chinese), a Kampo formula, is effective on vascular dementia clinically, and the hooks and stems of *Uncaria sinensis* (OLIV.) HAVIL., a medicinal plant comprising Choto-san, has a neuroprotective effect in vitro. In the present study, for the purpose of clarifying their effects in vivo, we investigated whether the oral administration of Choto-san extract (CSE) or *U. sinensis* extract (USE) reduces delayed neuronal death following ischemia/reperfusion (i/rp) in gerbils. Transient forebrain ischemia was induced by bilateral carotid artery occlusion for 4 min, and two doses (1.0% and 3.0%) of CSE or USE were dissolved in drinking water and provided to the gerbils ad libitum from 7 days prior to i/rp until 7 days after i/rp. It was found that 1.0% and 3.0% CSE treatments significantly reduced pyramidal cell death in the hippocampal CA1 region at 7 days post i/rp. Three percent USE treatment also inhibited pyramidal cell death significantly at 7 days after i/rp. Superoxide anion and hydroxyl radical scavenging activities of the homogenized hippocampus at 7 days after i/rp in the 1.0% CSE- and 3.0% USE-treated groups were significantly enhanced compared to those of control. Further, lipid peroxide and NO_2^-/NO_3^- levels of the homogenized hippocampus at 48 h after i/rp in the 1.0% CSE- and 3.0% USE-treated groups were significantly lower than those of control. These results suggest that the oral administration of CSE or USE provides a protective effect against transient ischemia-induced delayed neuronal death by reducing oxidative damage to neurons.

Keywords: Choto-san; *Uncaria sinensis*; Cerebral ischemia; Reperfusion; Hippocampus; Neuroprotection

Introduction

The brain is particularly susceptible to an insufficiency of blood supply. Permanent or transient ischemia is a critical factor in the appearance of brain injury, and it is induced at least in part by the toxicity of reactive oxygen species (White et al., 2000; Bolanos and Almeida, 1999).

During brain ischemia, excessive amounts of glutamate are released from vesicles in pre-synaptic neurons, overstimulating glutamate receptors in post-synaptic neurons, especially *N*-methyl-D-aspartate receptors

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(White et al., 2000). This event leads to excessive Ca^{2+} influx into neurons through voltage-dependent and more glutamate-regulated Ca^{2+} channels (Lipton and Rosenberg, 1994), followed by the activation of the Ca^{2+}-dependent neuronal form of nitric oxide (NO) synthase and the subsequent production of NO free radicals (NO•) (White et al., 2000). In the later phase of reperfusion, several stimuli involving activation of some kinds of cytokines strongly induce the Ca^{2+}-independent inducible form of NO synthase (iNOS) expression in glial cells, allowing excessive and uncontrolled production of NO·(Bolanos and Almeida, 1999). In this process, large amounts of superoxide anions (O_2^-•) are generated as a result of mitochondrial dysfunction, accumulation of hypoxanthine (HPX), release of arachidonic acid, activation of leukocytes, and so forth (White et al., 2000). Increasing evidence from in vitro studies suggests that the endogenous formation of peroxinitrate anion ($ONOO^-$), from the reaction of NO• with O_2^-, and further hydroxyl radical (HO•) generation, may be a possible mechanism by which neurotoxiciy is induced (Lafon-Cazal et al., 1993). Knowledge of these pathophysiological mechanisms has enabled investigators to develop successful therapeutic strategies against brain injury induced by ischemia/reperfusion (i/rp) in animal experiments, such as glutamate receptor antagonists (Ishimaru et al., 1997), NO synthase (NOS) inhibitors (Kohno et al., 1996), antioxidants/free radical scavengers (Bagenholm et al., 1996; O'Neill et al., 1997), and so on.

Choto-san (Diao-Teng-San in Chinese), a traditional Chinese/Japanese (Kampo) formula, has been administered to relatively aged patients with physical weakness and such subjective symptoms as headache, dizziness, vertigo, tinnitus, and so forth in Japan. Many of these symptoms are thought to originate from disorders in the cerebrovascular system. Recently, we demonstrated the effectiveness of Choto-san on patients with vascular dementia by well-controlled and double-blind studies (Shimada et al., 1994; Terasawa et al., 1997). Uncaria Uncus Cum Ramulus originating from the hooks and stems of *Uncaria sinensis* (OLIV.) HAVIL. is regarded as the main medicinal plant comprising Choto-san. We also recently revealed that the *U. sinensis* water extract and its phenolic and alkaloid compounds have protective effects against glutamate- and NO donor-induced neuronal death in cultured cerebellar granule cells (Shimada et al., 1998, 1999, 2001, 2002). However, the protective effect of the orally administered Choto-san or *U. sinensis* against brain injury induced by ischemia in vivo has not been sufficiently clarified.

It is well known that forebrain cerebral i/rp by transient occlusion of bilateral carotid arteries induces delayed neuronal death in the hippocampal CA1 region in the gerbil (Kirino, 1982). In the present study, using this animal model, we investigated the effects of orally administered water extracts of Choto-san and *U. sinensis* on delayed neuronal death in the hippocampal CA1 region, and also on O_2^-• and HO• scavenging activities and lipid peroxide (LPO) and nitrite (NO_2^-)/nitrate (NO_3^-) production in the hippocampus.

Methods

Preparation of Choto-san and *Uncaria sinensis* extract

Choto-san was composed of 11 kinds of crude drugs mixed in the ratios shown in Table 1. All of these crude drugs were purchased from Tochimoto Pharmaceuticals (Osaka, Japan). This mixture (total 100 g) was extracted with boiling water (500 ml) for 50 min. The solution was centrifuged at 10,000*g* for 30 min, and the supernatant was then converted to freeze-dried powder as Choto-san extract (CSE). The water extracts of *U. sinensis* extract (USE) and other constituents of Choto-san were obtained from 100 g of each of the dried crude drugs by the above procedure. The yields of Choto-san and each of the constituent extracts are shown in Table 2.

Table 1. The 11 crude drugs composing Choto-san and their weight ratios

		Weight (g)
Uncariae Uncis Cum Ramulus	Hooks and stem of *Uncaria sinensis* Haviland	3.0
Aurantii Nobilis pericarpium	Peel of *Citrus unshu* Markovich	3.0
Pinelliae tuber	Tuber of *Pinellia ternata* Breitenbach	3.0
Ophiopogonis tuber	Root of *Ophiopogon japonicus* Ker-Gawler	3.0
Poria	Sclerotium of *Poria cocos* Wolf	3.0
Ginseng radix	Root of *Panax ginseng* C.A. Meyer	3.0
Chrysanthemi flos	Flower of *Chrysanthemum morifolium* Ramatulle	3.0
Saphoshnikoviae radix	Root and rhizome of *Saposhnikovia divaricata* Schischkin	3.0
Glycyrrhizae radix	Root of *Glycyrrhiza uralensis* Fisher	1.0
Zingiberis rhizoma	Rhizome of *Zingiber officinale* Roscoe	1.0
Gypsum Fibrosum	$CaSo_4 \cdot 2H_2O$	5.0

Table 2. Yields and O_2^-• and HO• scavenging activities of extracts of Choto-san and the comprising crude drug

	Yield of extract (%)	IC50 (mg/ml)	
		O_2^-•	HO•
Choto-san	10.6	0.84	4.68
Uncariae Uncis Cum Ramulus	7.0	0.02	2.77
Aurantii Nobilis pericarpium	8.8	1.72	3.83
Pinelliae tuber	1.6	40.78	4.22
Ophiopogonis tuber	38.9	137.28	64.06
Poria	0.5	4.37	0.77
Ginseng radix	10.1	302.41	2.17
Chrysanthemi flos	6.2	0.55	4.12
Saphoshnikoviae radix	7.6	3.99	3.54
Glycyrrhizae radix	16.0	3.40	4.43
Zingiberis rhizoma	3.3	1.33	0.97
Gypsum Fibrosum	0.9	—	2.00

Analysis of 3D-HPLC fingerprints of CSE and USE

CSE and USE (0.5 g) were extracted with methanol (20 ml) under ultrasonication for 30 min. The solution was filtrated and then submitted to HPLC analysis.

HPLC equipment was controlled with an HPLC pump (LC-10AD; Shimadzu, Kyoto, Japan) using a TSK-GEL, ODS-80TS column (4.6φ × 250 mm), and elution was done with solvents (A) 0.05 M $AcONH_4$ (pH 3.6) and (B) CH_3CN. A linear gradient of 100% A and 0% B changing over 60 min to 0% A and 100% B was used. The flow rate was controlled with LC-10AD at 1.0 ml/min. The eluate from the column was monitored, and the three-dimensional data were processed with a diode array detector (SPD-M10A; Shimadzu, Kyoto, Japan). The three-dimensional HPLC charts of the methanol solutions of CSE and USE are shown in Figs. 1and 2, respectively.

Animals

Adult male Mongolian gerbils (10 weeks old, 60–65 g) were purchased from Japan FUB Corporation (Hamamatsu, Japan). They were kept in an animal room at an ambient temperature of 23 ± 1°C under a 12-h dark–light cycle. They were allowed an adaptation period of at least 1 week. They were operated after 12 weeks (60–65 g). All animal use procedures were approved by the Committee on Animal Experimentation of Toyama Medical and Pharmaceutical University.

Grouping and treatment

Two doses (1.0% and 3.0%) of CSE were dissolved in drinking water and the animals were given access ad libitum. The animals were randomly divided into the following four 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.

Surgery

Surgical procedures were performed according to the method of Kirino (1982) with slight modification. Gerbils were placed in an anesthetic container with 2.5% halothane and a mixture of nitrous oxide (50%) and oxygen (50%) for 5 min. During the surgical procedure, the anesthesic level was maintained with 1.0% halothane via a nose cone. The carotid arteries were exposed by a midline neck incision. After they were isolated, loosely looped around by silk threads, they were occluded for exactly 4 min with microaneurysm clips. Brain temperature was monitored by animal body temperature controller (ATB-1100, Nihon Kohden, Tokyo, Japan) with a temporalis muscle probe, and maintained at 37.0°C with a heating blanket and lamp. At the completion of the occlusion period, the clips were removed and reperfusion was visually confirmed before the neck was closed with silk threads. Sham surgeries were conducted in a similar manner, except that the carotid arteries were not occluded.

Histology

At 7 days after i/rp, the animals were deeply sedated by intraperitoneal injection of pentobarbital (0.1 ml, 65 mg/ml) and perfused with saline containing heparin (2 U/ml) followed by saline containing 10% paraformaldehyde. After perfusion, brains were removed, stored in paraformaldehyde solution for 7 days, and transferred to a solution containing 30% sucrose and 10% paraformaldehyde for 1 day. The brains were sliced into 20 μm sections using a freezing microtome at −30°C, and the sections were mounted on slides and stained with cresyl violet. Stained slices at the level of the dorsal hippocampus 2.0 mm posterior to bregma were analyzed for damage to the CA1 region.

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 (BH-2, Olympus, Tokyo, Japan) at × 400 magnification. Their total number was expressed as percentage of the average of sham-operated animals. The cell number was counted by an observer blinded to the various treatment groups.

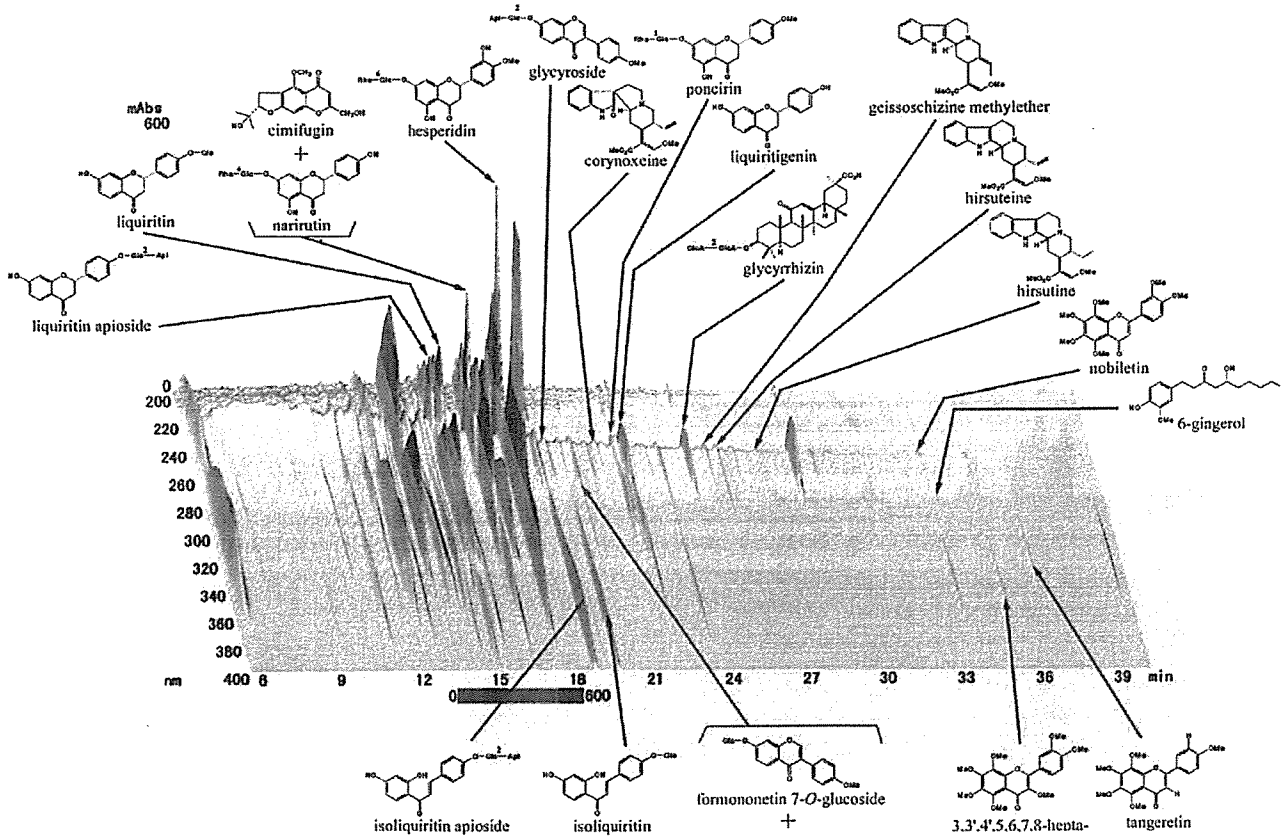

Fig. 1. Three-dimensional HPLC profile of the methanol solution of Choto-san water extract (CSE).

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 3000*g* for 10 min at 4°C, they were used for measurements of LPO, NO_2^-/NO_3^- and free radical scavenging activities against both $O_2^-\bullet$ and HO•.

Measurement of LPO

The LPO content in the homogenate supernatant of the hippocampus was measured by lipid peroxidation assay kit (Determina LPO; Kyowa Medex Co., Tokyo, Japan) according to the manufacturer's instruction.

Measurement of NO_2^-/NO_3^-

NO_2^-/NO_3^- content in the homogenate supernatant was measured by an automated system (ENO-10, EICOM CO., Kyoto, Japan), based on the Griess technique (Green et al., 1982).

Measurement of $O_2^-\bullet$ and HO• scavenging activities

Measurement of $O_2^-\bullet$ and HO• scavenging activities was performed as described previously (Ohsugi et al., 1999) with slight modification. After aliquots of the prepared hippocampal homogenate supernatant (50 µl) were diluted to 10-fold with PBS (pH 7.4), $O_2^-\bullet$ and HO• scavenging activities of these samples were assessed by electron spin resonance (ESR) technique (Buettner, 1987).

In this experiment, $O_2^-\bullet$ was generated from a HPX-xanthine oxidase (XOD) reaction system in PBS (Mitsuta et al., 1990). Briefly, 15 µl of 9.2 M 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (LABOTEC, Tokyo, Japan), 50 µl of 2 mM HPX (Sigma, St. Louis, USA), 35 µl of 5.5 mM diethylenetriamine-*N*,*N*,*N'*,*N''*,*N''*-pentaacetic dianhydride (DETAPAC; Wako Pure Chemical Industries, Tokyo, Japan) and 50 µl of prepared sample were put into a test tube. After adding

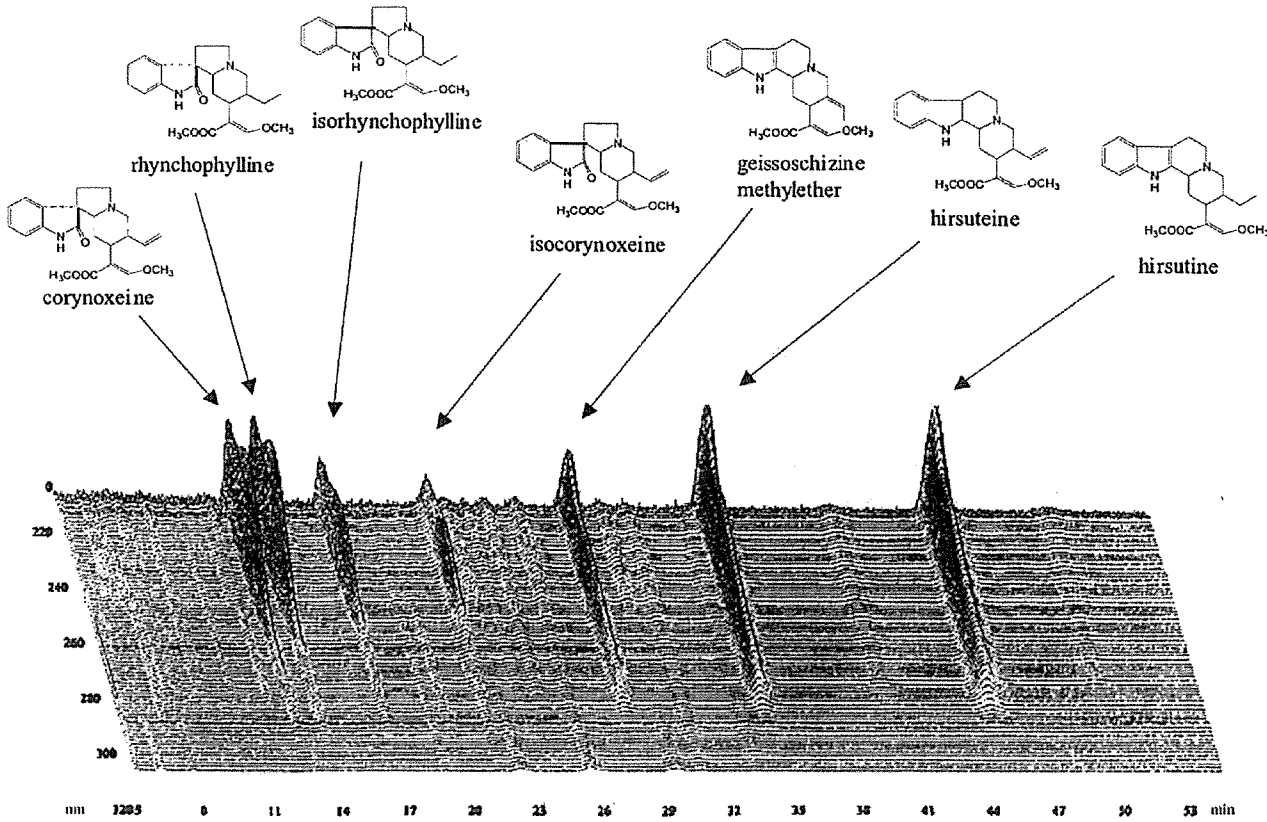

Fig. 2. Three-dimensional HPLC profile of the methanol solution of *U. sinensis* water extract (USE).

50 µl of 0.4 U/ml XOD (Roche, Indianapolis, USA) and quick mixing, 200 µl of the mixture was transferred to a flat quartz ESR cuvette, which was fixed to the cavity of an ESR spectrometer (JES-FR30, JOEL, Tokyo, Japan). Recordings of the spectra were made at 24°C and started at 1 min after the mixing of XOD; each scan took 1 min. Data were expressed as the ratio of the peak of the DMPO-OOH signal to the peak of the intrinsic standard, MnO (S/M). The scavenging activity (Scv) was calculated by the following equation:

$$\text{Scv}\ (\%) = \frac{\text{S/M}_{\text{BLANC}} - \text{S/M}_{\text{SAMPLE}}}{\text{S/M}_{\text{BLANC}}} \times 100,$$

where S/MBLANC is the intensity of the ESR spectrum of DMPO-OOH spin adduct in PBS as a blanc, and S/MSAMPLE is the intensity of the ESR spectrum of DMPO-OOH spin adduct in the sample.

HO• was generated by the Fenton reaction (Kohno et al., 1991) consisting of 75 µl of 0.1 mM H_2O_2, 50 µl of each sample, 20 µl of 92 mM DMPO and 75 µl of 0.1 mM $FeSO_4$. The spectrum of DMPO-OH was measured at 1 min after the addition of H_2O_2. Scavenging activity was calculated as described above.

In addition, the water extracts of Choto-san and its constituents were diluted to 0.2, 2.0, and 20.0 mg/ml with PBS. O_2^-• and HO• scavenging activities of each sample (50 µl) were assessed by ESR technique, and then IC50 (inhibition concentration 50%) was calculated.

Statistical analysis

Values were expressed as mean ± S.E. The data were analyzed by one-way analysis of variance followed by Fisher's PLSD. A p-value <0.05 was considered statistically significant.

Results

In the present study, body weight did not differ among any of the groups at the time points of starting administration, surgery and sacrifice (data not shown). Water consumption also did not vary (data not shown), as 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 or USE groups ingested approximately 1.0 and 3.0 g/kg/day of CSE or USE, respectively.

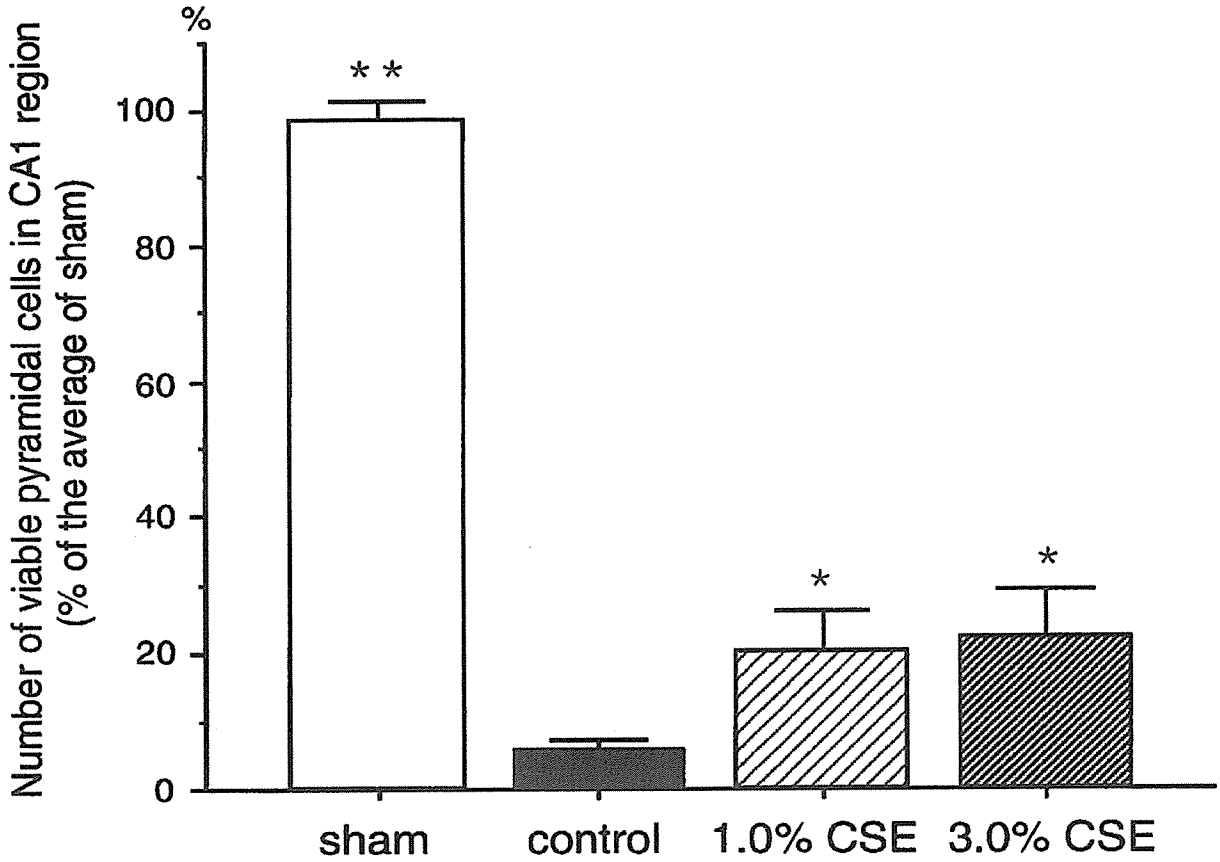

Fig. 3. Numbers of viable pyramidal cells in hippocampal CA1 region at 7 days after i/rp; sham-operated group (sham), i/rp without treatment group (control), i/rp with 1.0% CSE-treated group (1.0% CSE), i/rp with 3.0% CSE-treated group (3.0% CSE). Each column represents the mean ± S.E. of 10 animals. $^{*}p<0.05$, $^{**}p<0.01$ compared with control.

Delayed neuronal death

In this animal model, neuronal damage occurred in the hippocampal CA1 region selectively, and no histological changes were observed in other areas of the brain.

Histological examination revealed that the number 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. 3). 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. 4). Typical photographs of the hippocampi of these groups are shown in Fig. 5.

O_2^- • and HO • scavenging activities of homogenized hippocampus

We used the oral administration of 1.0% CSE and 3.0% USE in order to determine if they enhanced the O_2^- • and HO • scavenging activities in the hippocampus.

The O_2^- • and HO • scavenging activities of the homogenized hippocampus obtained from gerbils not undergoing i/rp after 7 days of continuous oral administrations of both of 1.0% CSE and 3.0% USE were significantly higher than those of non-treated control (Table 3).

The O_2^- • and HO • scavenging activities of the homogenized hippocampus at 7 days after i/rp in both 1.0% CSE and 3.0% USE groups, administered drugs from 7 days prior until 7 days after i/rp, were significantly higher than those of control (Table 4).

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 h after i/rp, were significantly lower than those of control (Fig. 6A and B).

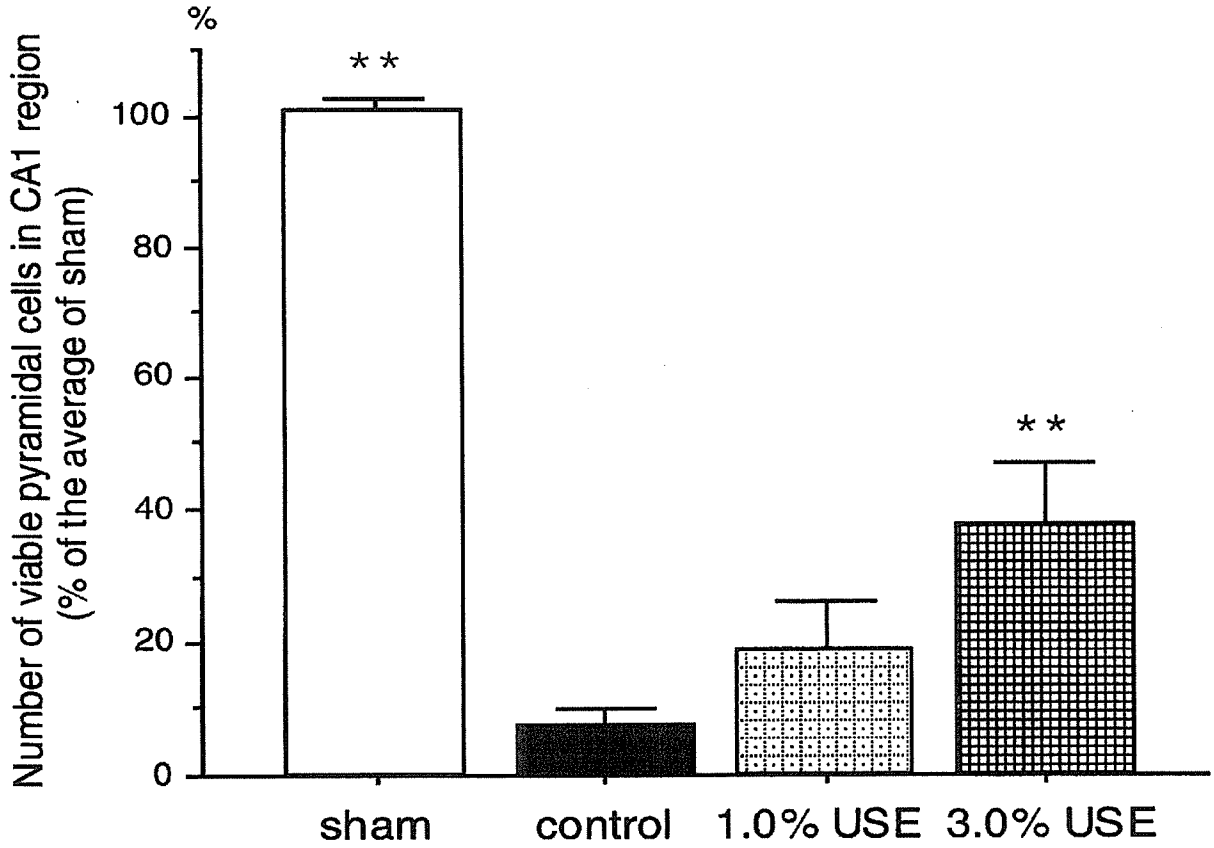

Fig. 4. Numbers of viable pyramidal cells in hippocampal CA1 region at 7 days after i/rp; sham-operated group (sham), i/rp without treatment group (control), i/rp with 1.0% USE-treated group (1.0% USE), i/rp with 3.0% USE-treated group (3.0% USE). Each column represents the mean ± S.E. of 10 animals. $^{**}p < 0.01$ compared with control.

O_2^- • and HO • scavenging activities (IC50) of extracts

Free radical scavenging activities (IC50) of CSE and each water extract of the crude drugs comprising Choto-san are shown in Table 2. CSE and USE have high scavenging activities against both O_2^- • and HO •. Other extracts, especially from Poria, Chrysanthemi flos and Zingiberis rhizoma, have relatively high scavenging activities against O_2^- • and HO •.

Discussion

In the present study, the oral administrations of CSE and USE to gerbils prevented delayed neuronal death of the hippocampal CA1 region induced by transient forebrain ischemia. This suggests that *U. sinensis* mainly contributes to the neuroprotective effect of Choto-san. Although we observed a tendency of higher-dose treatment to be more effective than lower-dose treatment, a distinctly dose-dependent effect was not evident. A ceiling effect might be present, although other concentrations need to be examined.

In general, it is well recognized that permanent or transient ischemia is a critical factor in the appearance of brain injury, and at least in part the toxicity of reactive oxygen species is responsible for the injury (White et al., 2000; Bolanos and Almeida, 1999). Consequently, antioxidants/free radical scavengers are considered to be effective therapeutic agents. Especially in natural products, it is known that phenolic compounds possess antioxidant and free radical scavenging properties (Plumb et al., 1998). An in vitro study showed that the phenolic compounds of red wine constituents can inhibit neuronal damage from the oxidative stress produced by NO generation (Bastianetto et al., 2000). In vivo studies have revealed that oral administrations of catechin (Inanami et al., 1998), green tea extract (containing catechin) (Hong et al., 2001) and *Ginkgo biloba* extract (containing ginkgo-flavonol glucosides, terpene lactones and procyanidines) (Calapai et al., 2000) provided protective effects against brain injury

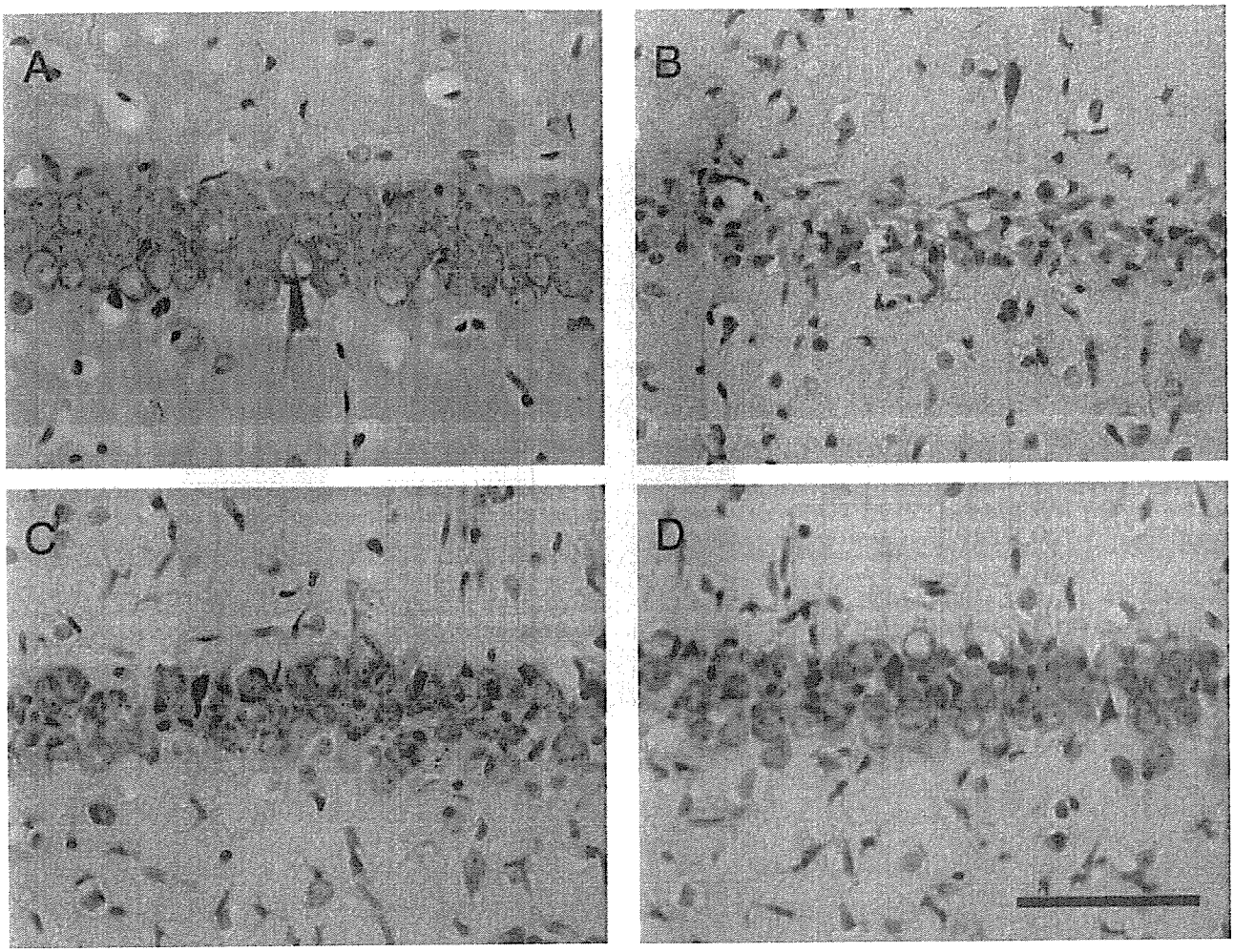

Fig. 5. Protective effect of CSE and USE on delayed neuronal death. Representative photographs of pyramidal cells in hippocampal CA1 region of sham-operated group (A, sham), i/rp without treatment group (B, control), i/rp with 3.0% CSE-treated group (C, 3.0% CSE) and i/rp with 3.0% USE-treated group (D, 3.0% USE) at 7 days after i/rp. Scale bar indicates 100 μm.

Table 3. O_2^-• and HO• scavenging activities of homogenized hippocampus obtained from gerbils without ischemia/reperfusion after 7 days of Choto-san extract (1.0% CSE) and 3.0% *Uncaria sinensis* extract (3.0% USE)

	Control	1.0% CSE	3.0% USE
O_2^-• scavenging activity (%)	27.1±1.0	42.6±1.1**	34.6±2.7**
HO• scavenging activity (%)	10.8±3.2	24.4±1.4**	22.3±2.7**

Mean±S.E., $n = 8$, **$p<0.01$ compared with control.

Table 4. O_2^-• and HO• scavenging activities of homogenized hippocampus obtained from gerbils 7 days after ischemia/reperfusion of Choto-san extract (1.0% CSE) and 3.0% *Uncaria sinensis* extract-treatment groups (3.0% USE)

	Control	1.0% CSE	3.0% USE
O_2^-• scavenging activity (%)	32.6±2.1	44.1±1.4**	41.1±1.3**
HO• scavenging activity (%)	13.8±1.5	20.4±1.0**	23.0±1.9**

Mean±S.E., $n = 8$, **$p<0.01$ compared with control.

induced by transient forebrain ischemia in gerbils. *U. sinensis* also contains phenolic compounds, such as epicatechin, catechin, procyanidin B-1, procyanidin B-2, hyperin and caffeic acid (Shimada et al., 2001). Other investigators reported that Uncaria genus has antioxidant and free radical scavenging activities in vitro and

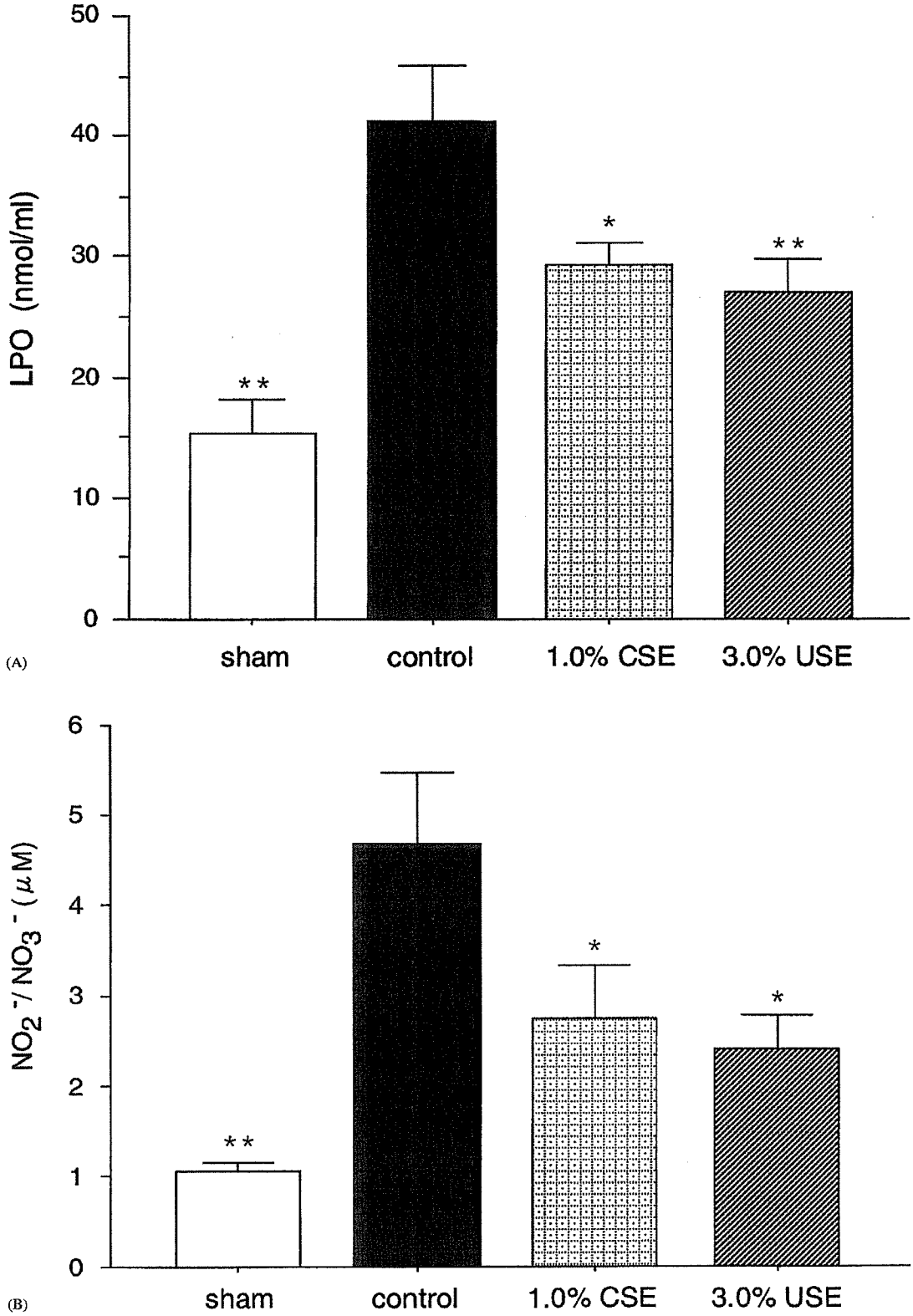

Fig. 6. LPO (A) and NO_2^-/NO_3^- (B) levels in homogenized hippocampus at 48 h after i/rp; sham-operated group (sham), i/rp without treatment group (control), i/rp with 1.0% CSE-treated group (1.0% CSE), i/rp with 3.0% USE-treated group (3.0% USE). Each column represents mean ± S.E. of eight animals. $^*p<0.05$, $^{**}p<0.01$ compared with control.

in vivo. From in vitro experiments, *Uncaria rhynchophylla* (MIQ.) JACK., which contains similar components to *U. sinensis*, was reported to possess free radical scavenging activities when examined by ESR technique (Ohsugi et al., 1999; Liu and Mori, 1992). In vivo experiments using *U. rhynchophylla* showed increased antioxidant activity and inhibited lipid peroxidation in the brain of ferric chloride-induced epileptic rats (Liu and Mori, 1992), and also decreased the lipid peroxide level in the brain of kainic acid-induced epileptic rats (Hsieh et al., 1999).

In the present study, it was revealed that O_2^-• and HO• scavenging activities of the hippocampal homogenate obtained from gerbils not only without i/rp procedure but also 7 days after i/rp were enhanced by oral administrations of 1.0% CSE and 3.0% USE. The data obtained from the cortex showed similar results (data not shown). In addition, the LPO levels of homogenized hippocampus in 1.0% CSE- and 3.0% USE-treated groups at 48 h post i/rp were lower than those of control. One of the mechanisms for these results being obtained may be free radical scavenging activities of CSE and USE themselves. Another may be the induction of endogenous antioxidants such as superoxide dismutase and glutathione peroxidase. Moreover, there are some possibilities that inhibition of NOS, an antagonistic effect on the glutamate receptors of neurons, and so on, might be involved. Further studies focusing on the clarification of these possible involvements need to be performed.

U. sinensis also contains alkaloid compounds, such as oxyindole alkaloids, corynoxeine, rhynchophylline, isorhynchophylline and isocorynoxeine, and indole alkaloids, geissoschizine methyl ether, hirsuteine and hirsutine (Shimada et al., 1999; Sakakibara et al., 1998). Rhynchophylline, isorhynchorhylline, corynoxeine, isocorynoxeine and hirsutine exhibit vasodilative and Ca^{2+} channel blocking activity in isolated rat thoracic aorta (Horie et al., 1992). Hirsuteine noncompetitively antagonizes nicotine-evoked dopamine release by blocking ion permeation through nicotinic receptor channel complexes in rat pheochromocytoma PC12 cells (Watano et al., 1993). Geissoschizine methyl ether decreases specific [^{3}H] 5-hydroxytryptamine binding to membrane preparations from rat brain (Kanatani et al., 1985). Further, the oral administration of geissoschizine methyl ether or hirsuteine inhibits glutamate-induced convulsion in mice (Mimaki et al., 1997). It was also reported that Uncaria genus, such as *U. sinensis* and *U. rhynchophylla*, has protective effects against neurotoxicity induced by excitatory amino acids in vivo (Mimaki et al., 1997; Hsieh et al., 1999) and in vitro (Shimada et al., 1998, 1999). Moreover, it was recently reported that intraperitoneal injection of methanol extract of *U. rhynchophylla* protected hippocampal CA1 neurons against transient forebrain ischemia in a 4-vessel-occlusion rat model, by anti-inflammatory effect such as inhibition of COX-2 expression (Suk et al., 2002). These reports suggest that, besides the antioxidative effect, several other effects of *U. sinensis*, such as Ca^{2+} channel and receptor blocking, together with anti-inflammatory effect, might contribute to its neuroprotective ability.

In our recent in vitro study, USE exerted a protective effect against NO donor-induced neuronal death in cultured cerebellar granule cells (Shimada et al., 2002), suggesting that USE works in a protective manner against NO-medicated neurotoxicity. In the present study, we observed a reduction in NO_2^-/NO_3^- levels in homogenized hippocampi from CSE- and USE-treated groups at 48 h post i/rp. From this, we can suppose that CSE and USE might exert an inhibitory effect on the induction of iNOS production or its activation, although it may also result from an anti-inflammatory effect by a reduction of the initial oxidative damage. Further studies will need to focus on the effect of USE and CSE on iNOS induction.

We also recently demonstrated that phenolic and alkaloid fractions/compounds of USE suppressed vasocontraction induced by oxidative stress (Goto et al., 2000), and we showed that the oral administration of USE had a protective effect on the endothelial function of spontaneously hypertensive rats (Goto et al., 1999). Taking these profiles of *U. sinensis* together, we are able to make a convincing argument about its beneficial effects on the pathophysiological mechanisms of brain ischemia.

In conclusion, the results of the present study seem to indicate that the oral administration of CSE or USE produces a neuroprotective effect against i/rp-induced brain injury possibly by the free radical scavenging effect. This and the previously revealed beneficial effects of Choto-san and *U. sinensis* on nervous and vascular systems strongly point to their potential use for the prevention of the development of ischemic cerebral diseases.

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Effects of two formulations for overcoming oketsu on vascular function and expression patterns of plasma proteins in spontaneously diabetic rats

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We investigated the effects of keishibukuryogan and tokishakuyakusan, which are representative formulations for overcoming oketsu, on vascular function and expression patterns of plasma proteins in spontaneously diabetic rats. Twenty-one- to 24-week-old male WBN/Kob rats were maintained for 18 weeks on a diabetes-accelerated feed, and received standard (diabetes-acccelerating) chow containing 3% (wt/wt) keishibukuryogan or tokishakuyakusan for 25 weeks. There was no significant change in body weight or blood glucose among the groups. Acetylcholine-induced endothelium-dependent relaxation of the keishibukuryogan group significantly increased compared to that of controls. Xanthine/xanthine oxidase-induced contraction of the tokishakuyakusan group and phospholipase A_2-induced contraction of the keishibukuryogan and tokishakuyakusan groups significantly decreased compared to the controls. Transit time of whole blood tended to decrease in the tokishakuyakusan group compared to controls. NO_2^-/NO_3^- in the keishibukuryogan and tokishakuyakusan groups significantly decreased compared to controls. A study using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) demonstrated that five and eight peaks had significantly changed peak intensities in plasma of rats treated with keishibukuryogan and tokishakuyakusan, respectively, as compared to the controls. Thus, two representative formulations for overcoming oketsu with different mechanisms of action had favorable effects against vascular dysfunction. Altered plasma protein levels were commonly observed in the rats administered these two formulations. Our study using ProteinChip technology may be useful for the evaluation of the relationship between the efficacy and the profiling of plasma protein expression after administration Kampo medicines, thus leading to the understanding of "Sho" in Kampo medicine.

Key words keishibukuryogan, tokishakuyakusan, vascular function, WBN/Kob rat, surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), protein chip.

Introduction

WBN/Kob rats are spontaneously diabetic rats that live for a long time with hyperglycemia. Several hyperglycemia-induced complications have been observed in WBN/Kob rats,[1)] including vascular dysfunction and diabetic nephropathy. This condition is called "oketsu," and is defined as a state of insufficient blood circulation and blood stasis resulting in diabetic retinopathy, etc. We have reported[2)] that this vascular dysfunction was improved by the Kampo medicine, keishibukuryogan. Tokishakuyakusan is also an important formulation for overcoming oketsu and has been reported to improve blood circulation.[3)] Clinically, keishibukuryogan and tokishakuyakusan are based on the oriental concept of Yin-yang and hypofunction-hyperfunction. However, the effects these formulations have on host function are not clear in terms of modern medicine. Furthermore, the differences in host responses are thought to affect variations in the "Sho" diagnosis in Kampo medicine.

In previous reports, we investigated the expression of genes and proteins associated with various diseases in order to clarify the scientific basis of "Sho" in Kampo medicine. Administration of the Kampo medicine hachimijiogan, which has been mainly used for fatigue, exhaustion, nephritis, and diabetes mellitus, was effective at reducing the expression of diabetic nephropathy but not at reducing blood glucose levels.[10)] The expression patterns of plasma proteins using ProteinChip array revealed that several proteins in the plasma may be involved in the development and/or progression of diabetic nephropathy in WBN/Kob rats and the efficacy of hachimijiogan.[10)]

In the present study, we investigated the effects of keishibukuryogan and tokishakuyakusan on vascular function in spontaneously diabetic rats. We also examined the expression patterns of plasma proteins in these rats using

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ProteinChip technology to identify multiple biomarkers associated with "Sho" diagnosis.

Materials and Methods

Animals. Twenty-one to 24-week-old male WBN/Kob rats obtained from Sankyo Labo Service (Toyama, Japan) were used. They were kept in an animal room at an ambient temperature of 23 ± 1 ℃ under a 12-h light-dark cycle. They were maintained for 18 weeks on a feed that accelerated diabetes (Labo MR-DBT, Nosan Corporation, Yokohama, Japan). Their diabetes was confirmed when their fasting blood sugar reached above 200 mg/dl.

Experimental protocols met the "Guidelines for Animal Experimentation" approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society.

Drugs. Powdered keishibukuryogan and tokishakuyakusan were purchased from Uchida Wakanyaku (Tokyo, Japan). Keishibukuryogan consisted of equal amounts of the following five crude drugs: 5g of Cinnamoni Cortex (*Cinnamomum cassia* BLUME), 5g of Hoelen (*Poria cocos* WOLF), 5g of Moutan Cortex (*Paeonia suffruticosa* ANDREWS), 5g of Persicae Semen (*Purnus persicae* BATASCH), and 5g of Paeonia Radix (*Paeonia lactiflora* PALL) in 25 g of keishibukuryogan. Tokishakuyakusan consisted of the following six crude drugs: 6 g of Alismatis Rhizoma (*Alisma orientale* JUZEPCZUK), 5 g of Paeoniae Radix (*Paeonia lactiflora* PALL), 4 g of Atractylodis Rhizoma (*Atractylodes lancea* DE CANDOLLE), 4 g of Hoelen (*Poria cocos* WOLF), 3 g of Cnidii Rhizoma (*Cnidium officinale* MAKINO), and 3 g of Angelicae Radix (*Angelica sinensis* DIELS) in 25 g of tokishakuyakusan.

Drug treatment. WBN/Kob rats were randomly assigned to three groups (control, 3% keishibukuryogan, 3% tokishakuyakusan). Rats in the control group received standard (diabetes-accelerating) chow for 25 weeks. Rats in the 3% keishibukuryogan and 3% tokishakuyakusan groups received standard chow containing 3% (wt/wt) keishibukuryogan and tokishakuyakusan for 25 weeks, respectively. In terms of total chow volume, the drug doses used in this study corresponded to about 10 times the clinical doses, respectively.

Body weight and blood sugar measurement. Body weight and blood glucose were measured at two-week intervals from the baseline period until sacrifice. Blood glucose was determined using commercial reagents (Glucose CII-Test Wako obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan).

Relaxation experiments. The rats were anesthetized (50 mg/kg i.p. pentobarbiturate) and sacrificed by drawing blood from the heart. A section of the thoracic aorta was carefully cleaned of fat and connective tissues, and 3-mm ring preparations were made. The rings were mounted on steel hooks in a Magnus chamber (UC-5TD, Kishimoto, Kyoto, Japan). One end of the aorta was attached to a force-displacement transducer (UM-203, Kishimoto) so that its isometric contraction could be recorded (T-634, Niko Bioscience, Tokyo, Japan). Baths were filled with 5 ml of Krebs solution of the following composition (mM): NaCl 120, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5 and glucose 10.0. The solution was maintained at 37℃ and bubbled continuously with 5% CO_2 in O_2 at pH 7.4. The rings were equilibrated for 40 min at an initial resting tension of 1 g. During this time, the Krebs solution in the tissue bath was replaced every 15 min. The rings were then precontracted with 5×10^{-7} M noradrenaline (NA). For endothelium-dependent relaxations, vessels were relaxed with acetylcholine (Ach) (10^{-9} to 10^{-4} M). To study the endothelium-independent relaxation of vascular smooth muscle, vessels were relaxed with sodium nitroprusside (SNP) (10^{-9} to 10^{-4} M). Relaxation was expressed as a percentage of the decrease in maximal tension obtained by NA-induced contraction.

Contraction experiments. *Contraction induced by xanthine/xanthine oxidase.* To determine the endothelium-dependent contraction of the aorta induced by oxygen-derived free radicals, we placed a segment of an aorta in medium containing xanthine (10^{-4} M) and 10^{-4} M N^G-nitro-L-arginine methylester (L-NAME). Oxygen-derived free radical-induced endothelium-dependent contraction of aortas was determined by the addition of 10 mU/ml xanthine oxidase to medium containing xanthine. This contraction was expressed as a percentage of the relative increase to the maximal tension obtained by 60 mM KCl-induced contraction.

Contraction induced by phospholipase A_2 (PLA_2). To examine the effect against thromboxane A_2 - and prostaglandin H_2-induced contraction, PLA_2 (1 U/ml) was administered and transient contraction was induced in medium containing 10^{-4} M L-NAME. The contraction was expressed as a percentage of 60 mM KCl maximum contraction.

Contraction induced by angiotensin II (Ang II). To examine the effect against Ang II-induced contraction, Ang II (10^{-8} to 10^{-6} M) was administered and transient contraction was induced in medium containing 10^{-4} M L-NAME. The contraction was expressed as a percentage of 60 mM KCl maximum contraction.

Measurement of plasma triglyceride, lipid peroxides, fibrinogen and nitric oxide (NO). Triglyceride was determined by the standard method. Lipid peroxides were measured by the Yagi method.[4] Plasma fibrinogen was measured by the thrombin time method.[5] NO is an extremely unstable molecule and rapidly undergoes oxidative degradation to the stable inorganic nitrogen oxides NO_2^-/NO_3^-, which were used here as indices of in vivo NO generation. Serum NO_2^-/NO_3^- was measured with an automated system (ENO-10; EICOM Co., Kyoto, Japan) based on the Griess reaction method.

Microchannel array flow analysis. The transit time of whole blood through the microchannel array was measured and used as a marker of blood fluidity. The detailed procedures and apparatus of the microchannel analysis (Microchannel Array Flow Analyzer [MC-FAN], type KH-2, Hitachi Haramachi Electronics Co. Ltd., Hitachi, Tokyo, Japan) have been described previously.[6] Briefly,

microgrooves formed in the surface of a single crystal silicon substrate were converted to leak-proof microchannels by covering them tightly with an optical flat glass plate. The microgrooves in the silicon microchannel chip resembling the size of capillaries (Bloody-6-5, 8736 channels; width, 5 μm; depth, 4.5 μm; length, 20 μm - Hitachi Haramachi Electronics Co. Ltd., Hitachi, Tokyo) were prefilled with saline. Heparinized whole blood samples were forced to flow through the microchannels under a pressure difference of 20 cm H_2O. To assess the filterability of whole blood, the transit time for 100 μl of blood was determined. These measurements were performed immediately after blood sampling at room temperature between 20℃ and 25℃. MC-FAN was calibrated with saline before each new measurement. Blood passage through an individual channel was observed and recorded using a video microscope system (WAT-231S, WATEC, Tokyo, Japan).

Plasma sample preparation for SELDI protein profiling. Plasma samples were centrifuged at 3000 rpm for 10 min to remove insoluble debris and stored at -80℃ until used in the SELDI profiling study. Samples were thawed and then diluted (1:10 v/v in 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholyte).

SELDI protein profiling. Various chip properties (hydrophobic, anionic, cationic, and metal binding) were initially evaluated to determine which affinity chemistry provided the best serum profiles in terms of number and resolution of proteins. The cationic CM10 Chip Array (Ciphergen Biosystems, Fremont, CA) produced the best results. Protein Chip Arrays were assembled into a deep-well type bioprocessor assembly (Ciphergen Biosystems) equipped in a Laboratory Automation Workstation Biomek® 2000 (Beckman Coulter, Fullerton, CA). Prior to sample loading, CM10 Arrays were equilibrated with 150 μl of buffer (100 mM sodium acetate, pH 5.0) into each well and then prewashed two times for 5 min on a shaker at room temperature. To the arrays, 90 μl of buffer (100 mM sodium acetate, pH 5.0) and 10 μl of diluted sample were added into each well, and then the arrays were incubated for 30 min on a shaker at room temperature, and washed three times with 150 μl of buffer for 5 min on a shaker at room temperature. After rinsing two times with 200 μl of deionized water, the arrays were removed from the bioprocessor assembly and air-dried. One microliter of 50% solution of the energy-adsorbing molecule (EAM): alpha-cyano-4-hydroxy cinnamic acid (CHCA) (Ciphergen Biosystems) in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid was applied two times onto each ProteinChip Array, letting the array surface air-dry between each CHCA application.

The protein chip arrays were analyzed using a ProteinChip Biology System Reader (Model PBS-IIc; Ciphergen Biosystems). Spectra were collected at a laser intensity of 165 and a detector sensitivity of 6 in the positive ion mode. The protein masses were calibrated externally using purified peptide and protein standards (Ciphergen Biosystems). A mass range from 2000-10000 Da was selected for analysis because this range contained the majority of the resolved protein/peptides. Molecular masses from 0-2000 Da were eliminated from analysis because this area contains adducts of EAM and possibly other chemical contaminants.

Data analysis. Data on vascular function and blood samples are presented as mean ± standard error (S.E.). Statistical comparisons were performed using Fisher's PLSD test and repeated measures ANOVA. The level of statistical significance was defined as $p<0.05$. Spectra were analyzed with ProteinChip Software (Version 3.2.0; Ciphergen Biosystems). In order to use the intensities as indicators of the relative abundance of peptide in the sample, baselines had to be subtracted and the intensities normalized. Normalization was performed by total ion current normalization function following the software instructions. Biomarker Wizard (Ciphergen Biosystems) was then used to identify corresponding peaks in each spectrum within 0.3% of the mass. The signal-to-noise ratio was set to 2 for the first pass and to 2 for the second pass. We used the Mann-Whitney *U* test for nonparametric data sets to compare the peak intensities of the protein profiling results from the different groups.

Results

Effect of keishibukuryogan and tokishakuyakusan on vascular functions in spontaneously diabetic rats. There were no discernible differences in the body weight or blood glucose among the control, keishibukuryogan and tokishakuyakusan groups (Table 1). As shown in Figure 1A, Ach-induced endothelium-dependent relaxation, reaching 10^{-6} M, and relaxation of the keishibukuryogan group were significantly increased as compared to the control

Table 1 Characteristics of WBN/Kob rats in different experimental groups.

Group		Control	Keishibukuryogan	Tokishakuyakusan
Body weight	(g)	360 ± 11.4	333 ± 11.2	332 ± 7.2
Blood sugar	(mg/dl)	736 ± 32.0	590 ± 74.5	655 ± 44.0
Triglyceride	(mg/dl)	202 ± 38.1	163 ± 25.9	155 ± 30.4
Lipid peroxide	($\times 10^{-6}$M)	5.0 ± 2.8	6.1 ± 1.6	6.2 ± 2.8
Fibrinogen	(mg/dl)	287 ± 34.3	278 ± 29.6	265 ± 21.8
Serum No_2^-/No_3^-	($\times 10^{-5}$M)	20.3 ± 2.21	9.8 ± 1.15[a]	7.7 ± 0.81[a]
Microchannel transit time of whole blood	(sec)	69.0 ± 1.0	65.6 ± 2.0	64.5 ± 1.9[b]

Each value is mean ± S.E. of 7 rats. [a]$p<0.05$, [b]$p<0.1$ vs WBN/Kob control group.

group ($p<0.05$). Maximum relaxations were 45.9 ± 1.1%, 59.7 ± 1.3% and 52.0 ± 1.2 % in control, keishibukuryogan and tokishakuyakusan groups, respectively (mean ± S.E., n=7). In endothelium-independent relaxation, there was no significant difference in SNP among the three groups (Fig. 1B).

Xanthine oxidase-induced contraction of the tokishakuyakusan group was significantly decreased as compared to the control group ($p<0.05$). The contractions at 10 mU/ml xanthine oxidase were 37.3 ± 3.2%, 28.6 ± 3.5% and 25.4 ± 3.8% in the control, keishibukuryogan and tokishakuyakusan groups, respectively (Fig. 2). PLA_2-induced contraction in both the keishibukuryogan and tokishakuyakusan groups was significantly decreased as compared to the control group ($p<0.05$). Contractions at 1 U/ml PLA_2 were 39.0 ± 3.8%, 27.5 ± 2.4% and 27.7 ± 1.4% in the control, keishibukuryogan and tokishakuyakusan groups, respectively (Fig. 2). Ang II-induced contraction in both the keishibukuryogan and tokishakuyakusan groups was significantly decreased as compared to the control group ($p<0.05$). Contractions at 10^{-6} M Ang II were 28.6 ± 2.6 %, 20.8 ± 2.8% and 20.1 ± 2.3% in the control, keishibukuryogan and tokishakuyakusan groups, respectively (Fig. 3).

Triglyceride, lipid peroxide and fibrinogen showed no significant differences among the control, keishibukuryogan and tokishakuyakusan groups. NO_2^-/NO_3^- decreased significantly in the keishibukuryogan and tokishakuyakusan groups compared to the control group. The effects of keishibukuryogan and tokishakuyakusan on blood fluidity were evaluated by the microchannel transit time of whole blood, measured by MC-FAN. Transit time of whole blood tended to decrease in the tokishakuyakusan group compared

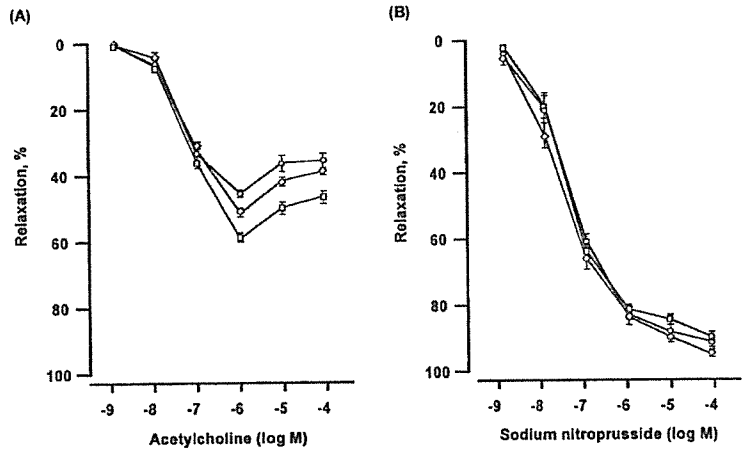

Figure 1 Graph showing A) endothelium-dependent relaxation in response to acetylcholine, B) endothelium-independent relaxation in response to sodium nitroprusside in the aorta of WBN/Kob rats treated for 25 weeks. WBN/Kob control group (○), keishibukuryogan group (□), and tokishakuyakusan group (◇). Values are expressed as a percentage of the decrease in maximal tension contracted with 5×10^{-7} M NA. Shown is the mean ± S.E. of seven determinations. Differences between WBN/Kob control and keishibukuryogan in graph (A) were statistically significant ($p<0.05$, n=7).

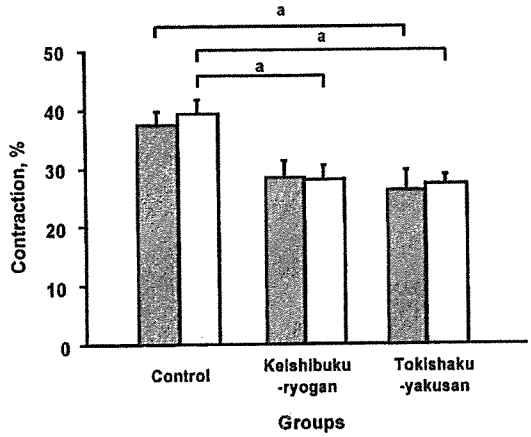

Figure 2 Vasocontraction treatment on xanthine oxidase (10 mU/ml) in the presence of xanthine (10^{-4}M) (■) and phospholipase (1 U/ml) (□) in the aorta of WBN/Kob rats treated for 25 weeks. All aortas had intact endothelium and had been treated with L-NAME. Contraction was expressed as a percentage of 60 mM KCl maximum contraction. Asterisks indicate significant differences from WBN/Kob control group ($^a p<0.05$, mean + S.E., n=7).

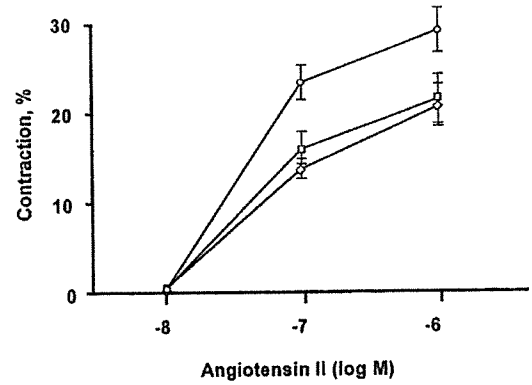

Figure 3 Graph showing angiotensin II induced-contraction in aorta of WBN/Kob rats treated for 25 weeks. WBN/Kob control group (○), keishibukuryogan group (□), and tokishakuyakusan group (◇). Contraction was expressed as the percentage of 60 mM KCl maximum contraction. Shown is the mean ± S.E. of seven determinations. Differences between WBN/Kob control and keishibukuryogan and tokishakuyakusan are statistically significant ($p<0.05$, n=7).

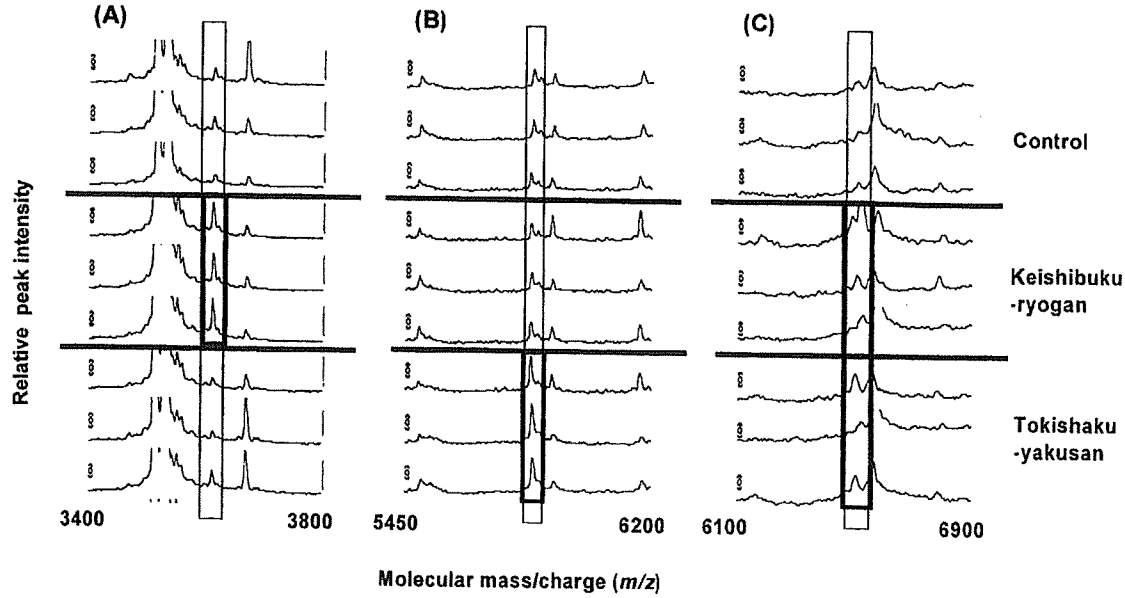

Figure 4 Typical peaks recognized by administration of keishibukuryogan and tokishakuyakusan. A) A typical peak elevated by administration of keishibukuryogan. B) A typical peak elevated by administration of tokishakuyakusan. C) A typical peak elevated by administration of both keishibukuryogan and tokishakuyakusan.

Table 2 Summary of *m/z* values and peak intensities changed significantly in the diabetic rats by keishiburyogan and tokishakuyakusan

	Peak Intensity (mean ± S.D.)		
m/z	Control	Keishibukuryogan	Tokishakuyakusan
3264.772	0.208 ± 0.102	0.309 ± 0.213	0.463 ± 0.400
3280.847	1.296 ± 0.187	1.138 ± 0.210	1.274 ± 0.372
3612.813	8.356 ± 2.445	11.998 ± 4.389	8.844 ± 3.864
3899.419	0.957 ± 0.146	1.022 ± 0.335	1.256 ± 0.183
4433.000	1.229 ± 0.302	1.506 ± 0.459	1.722 ± 0.623
4459.540	0.364 ± 0.142	0.519 ± 0.128	0.524 ± 0.185
4490.796	0.114 ± 0.093	0.221 ± 0.096	0.193 ± 0.104
5837.196	3.937 ± 0.696	4.124 ± 0.887	5.006 ± 0.690
5854.691	1.642 ± 0.202	1.742 ± 0.249	1.835 ± 0.203
6506.587	1.149 ± 0.313	1.385 ± 0.280	1.618 ± 0.665

Underline : $p<0.05$ vs. control by Mann-Whitney *U* test.

to the control group (Table 1).

Changes in plasma protein profiling of diabetic rats after oral administration of keishibukuryogan and tokishakuyakusan. We investigated the influence of keishibukuryogan and tokishakuyakusan on the expression patterns of plasma proteins in WBN/Kob rats at 67 weeks of age. Spectral analysis of samples (control group, n=7; keishibukuryogan group, n=7; tokishakuyakusan group, n=7) was performed in duplicate using the ProteinChip software program. Approximately 230 peaks per spectrum were detected in the 2000-10000 Da mass range. Representative spectra of plasma proteins of WBN/Kob rats are shown in Fig. 4. As summarized in Table 2, administration of keishibukuryogan led to significant changes in the intensities of the five peaks (*m/z* 3280, 3612, 4459, 4490 and 6506), and that of tokishakuyakusan led to significant elevation in the intensities of eight peaks (m/z 3264, 3899, 4433, 4459, 4490, 5837, 5854, and 6506), compared with control diabetic rats. Three of the peaks (m/z 4459, 4490 and 6506) were elevated by the administration of both keishibukuryogan and tokishakuyakusan.

Discussion

The WBN/Kob rat is a spontaneously insulin-dependent diabetic rat, in which the diabetes is caused by pancreatitis. This animal has a long life span, so several hyperglycemia-induced complications have been observed.[1] Several vascular dysfunctions have also been observed in WBN/Kob rats, such as endothelial dysfunction.[7] In this regard, some Kampo formulations for overcoming oketsu have been used to treat gynecological, psychiatric, and dermatological diseases *etc.* For arteriosclerosis, vascular protective effects that improve blood circulation have been reported for formulations used for overcoming oketsu.[8,9] Recently, we demonstrated that keishibukuryogan improved vascular dysfunction in WBN/Kob rats through improvement of endothelial function, suppression of vasocontraction, and decreasing blood viscosity.[2]

In the present study, we examined the effects of tokishakuyakusan in WBN/Kob rats in comparison with the effects of keishibukuryogan. The results were that keishibukuryogan was effective at improving endothelial function more than tokishakuyakusan, while tokishakuyaku-

san was effective at improving blood fluidity more than keishibukuryogan. Because the different Kampo formulations caused different responses in vascular function even in the same model, it is thought that the responses of the body also vary according to the various kinds of Kampo formulations. This variety in body responses is explained by the Kampo diagnosis of "Sho".

Since Kampo formulations are generally prepared from the combination of several crude drugs, these drugs are believed to have harmonization effects, which results in different effects than each individual crude drug. Therefore, in order to evaluate the influence of Kampo formulations on various diseases that result from multiple factors, an inclusive analytical method, such as ProteinChip technology, may be useful for profiling biological mixtures and identifying multiple biomarkers associated with diseases. We recently reported that hachimijiogan prevented renal dysfunction-induced hyperglycemia in WBN/Kob rat, and that several plasma proteins may be involved in the progression of disease and the efficacy of hachimijiogan.[10] Here we identify one of these proteins and evaluate the relationship between the efficacy of hachimijiogan and expression profiling.

The expression pattern of plasma proteins by SELDI profiling revealed that five peaks in the keishibukuryogan-treated group and eight peaks in the tokishakuyakusan-treated group were significantly changed in the 2000-10000 Da mass range compared to the control group (Table 2). Three peaks at *m/z* 4459, 4490 and 6506 were observed to be commonly elevated after the oral administration of keishibukuryogan and tokishakuyakusan. On vascular functions and blood fluidity particular effects were seen with keishibukuryogan and tokishakuyakusan. Further studies will be needed to examine the correlation between these effects and expression patterns of plasma proteins in detail. In Kampo medicine, keishibukuryogan is used for the patient who is Yang and hyperfunctional, while tokishakuyakusan is used for the patient who is Yin and hypofunctional. Thus, the differences of "Sho" for both medicines may be related to the differences in expression profiling of proteins in plasma as well as the mechanism of action.

Table 3 shows the summary of our present and previous studies on the expression patterns of plasma proteins in spontaneously diabetic rats after oral administration of three Kampo medicines using SELDI-TOF-MS. Administration of hachimijiogan significantly decreased the increased levels of six plasma proteins as compared with the control group.[10] These six plasma proteins associated with hachimijiogan were evidently different at the *m/z* level from those of formulations for overcoming oketsu, keishibukuryogan and tokishakuyakusan (Table 3). As increase in the intensities of three peaks at *m/z* 4459, 4490 and 6506 was commonly observed in both keishibukuryogan and tokishakuyakusan groups, the expression of these proteins may be specific or characteristic in the formulations for overcoming oketsu. These results may provide an important basis for clarifying "Sho" and the determination of "Sho"-directed formulations. On the other hand, shakuyaku and

Table 3 Changes in expression of plasma proteins of spontaneously diabetic WBN/Kob rats administered keishiburyogan, tokishakuyakusan and hachimijiogan

Group / *m/z*	Keishibukuryogan	Tokishakuyakusan	Hachimijiogan
3264		↑	
3280	↓		
3612	↑		
3899		↑	
4433		↑	
4459	↑	↑	
4490	↑	↑	
4679			↓
4733			↓
4808			↓
5837		↑	
5854		↑	
6506	↑	↑	
9058			↓
9323			↓
9465			↓

bukuryo are commonly contained in keishibukuryogan ar tokishakuyakusan; keishi, botanpi and bukuryo in keishib kuryogan and hachimijiogan; and takusha and bukuryo tokishakuyakusan and hachimijiogan. Thus, further stuc will be needed to examine whether the combination of cru drugs contained in the three formulations can be associat with the changes in expression patterns of plasma protei after administration of these formulations.

Acknowledgements

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Japanese abstract

自然発症糖尿病モデルであるWBN/Kobラットに代表的な駆瘀血薬である桂枝茯苓丸と当帰芍薬散を長期間投与し，血管機能とタンパク発現に及ぼす影響を検討した。方法は，WBN/Kobラット（雄，24週令）を18週間飼育し糖尿病発症を確認した後，対照群，3％桂枝茯苓丸（KB）群，3％当帰芍薬散（TS）群の3群に分け，さらに25週間飼育した。飼育後，胸部大動脈を摘出しOrgan bath法を用いacetylcholine（Ach）による血管弛緩作用，xanthine/xanthine oxidase（X/XOD）投与による血管収縮作用等を検討した。同時に，血液流動性，血漿脂質，NO代謝物等の測定とSELDI-TOF-MSによる血漿プロテオーム解析を施行した。結果は，対照群とKB，TS群の3群間において，体重と血糖値に有意な差を認めなかった。Achによる内皮依存性血管弛緩率はKB群で対照群に対し有意に弛緩率の増加を認めた。X/XOD投与による血管収縮率はTS群で，PLA_2投与による血管収縮率はTS，KB群の両群で対照群に対し収縮率の減少を認めた。血液流動性はTS群で対照群に対し改善傾向を認め，NO代謝物はKB，TS群の両群で対照群に対し有意に減少した。血漿プロテオーム解析により，対照群に比較しKB群では5個，TS群で8個のタンパク質の有意な変動を認めた。以上のことから，2種類の代表的な駆瘀血薬は，一部異なる作用機序で血流改善に影響を及ぼし，発現するタンパク質にも差異が認められた。作用機序とタンパク質発現との関連は今後検討を要するが，これらの多成分系の方剤による生体の複雑な反応性の差異が「証」の成立に影響していると考えられた。

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Cinnamaldehyde Induces Endothelium-Dependent and -Independent Vasorelaxant Action on Isolated Rat Aorta

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The vasorelaxant effect of cinnamaldehyde, one of the major oil components in Cinnamomi Cortex, was studied using isolated rat aorta. Cinnamaldehyde at final concentrations of 1 μM to 1 mM showed dose-dependent relaxation of the rat aorta contracted by treatment with prostaglandin $F_{2\alpha}$, norepinephrine or KCl. In addition, cinnamaldehyde relaxed prostaglandin $F_{2\alpha}$-precontracted aortic rings with endothelium and without endothelium, with the latter being significantly less sensitive than the former. Relaxation induced by cinnamaldehyde with endothelium was significantly inhibited by N^G-nitro-L-arginine methyl ester (L-NAME), while nonselective cyclooxygenase inhibitor (indomethacin), β-adrenergic receptor blocker (propranolol), an inhibitor of phosphodiesterase (theophylline), a delayed rectifier K^+ channel blocker (tetraethyl ammonium chloride), or an ATP-sensitive K^+ channel blocker (glibenclamide) did not reduce the relaxation induced by cinnamaldehyde with endothelium treated by L-NAME. Conversely, aorta pretreatment with L-NAME and theophylline increased the relaxation by cinnamaldehyde significantly compared to aorta pretreatment with only L-NAME. Furthermore, cinnamaldehyde significantly inhibited Ca^{2+}-induced contraction. These results suggested that the vasorelaxant effects of cinnamaldehyde were derived from both endothelium-dependent and -independent effects. Endothelium-dependent relaxation is affected by nitric oxide, and one of the mechanisms of endothelium-independent relaxation is thought to be influenced by the blocking of Ca^{2+} channels.

Key words cinnamaldehyde; endothelium-dependent relaxation; nitric oxide; endothelium-independent relaxation; theophylline; rat aorta

Cinnamomi Cortex is a crude drug used therapeutically in Asia and Europe. Its main component is cinnamaldehyde. There are many reports on the pharmacological effects of Cinnamomi Cortex. Mainly, the sedative effect of decreasing spontaneous motor activity,[1] anti-inflammatory effects related to cyclooxygenase-2,[2] and antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*[3] have been reported. In addition, in oriental medicine, Cinnamomi Cortex is often used to improve blood circulation. In regard to the circulatory system, a catecholamine-releasing effect of cinnamaldehyde,[4] a reducing effect on platelet aggregation due to suppression of the release of arachidonic acid from platelets,[5] and an inhibitory effect of collagen-induced platelet aggregation[6] have been reported. However, reports concerning the direct effect against vasomotion by components of Cinnamomi Cortex are few, except in relation to cinnamtannin.[7] In the present study, the vasorelaxant effects of cinnamaldehyde were studied by the organ bath method, and the mechanisms of vasorelaxation were evaluated.

MATERIALS AND METHODS

Drugs and Chemicals Analytical grades of the following reagents were purchased: cinnamaldehyde, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), norepinephrine (NE), acetylcholine, sodium nitroprusside dehydrate (SNP), methylene blue trihydrate, theophylline, propranolol hydrochloride, indometacin, tetraethylammonium chloride (TEA), glibenclamide and verapamil hydrochloride, all from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). N^G-nitro-L-arginine methylester (L-NAME) was from Sigma (St. Louis, MO, U.S.A.) and pentobarbital sodium salt was from Tokyo Chemical Ind. (Tokyo, Japan).

Animals Male Wistar rats weighing 370—420 g were purchased from Japan SLC (Shizuoka, Japan). They were kept in an animal room at an ambient temperature of 23±1 °C under a 12-h dark–light cycle. Experimental protocols met the "Guidelines for Animal Experimentation" approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society.

Preparation of Rat Aorta Rats were anesthetized (50 mg/kg i.p. pentobarbital) and sacrificed by cutting their abdominal aorta. Fats and connective tissues were removed from a section of the thoracic aorta, and 3-mm-wide aortic rings were prepared. For an endothelium-free aorta, the endothelial lining of each ring was removed by pressing the ring and rolling it gently onto a filter paper a few times. The endothelium was considered to be intact when relaxation induced by 1 μM of acetylcholine was over 20% of the maximal tension obtained by 60 mM KCl-induced contraction, and the removal of the endothelium was confirmed by the absence of acetylcholine-induced relaxation.

Vasodilative Effect of Cinnamaldehyde on Isolated Aortic Rings The aortic rings were mounted on steel hooks in a Magnus chamber (Kishimoto UC-5TD, Kyoto, Japan). One end of the aorta was attached to a force-displacement transducer (Kishimoto UM-203) so that its isometric contraction could be recorded (NEC RECTI-HORIZ-8K, Tokyo, Japan). The baths were filled with 5 ml of Krebs solution containing the following (mM): NaCl 120, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, and glucose 10.0. The solution was maintained at 37 °C and bubbled continuously with 5% CO_2 in O_2 at pH 7.4. The rings were equilibrated for 45 min at an initial resting tension of 1 g. During this period, the Krebs solution in the bath was

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