

Effects of Toki-shakuyaku-san on microcirculation of bulbar conjunctiva and hemorheological factors in patients with asymptomatic cerebral infarction

Qiao YANG,^{a)} Hirozo GOTO,^{*b)} Hiroaki HIKIAMI,^{c)} Naotoshi SHIBAHARA,^{b)} Yutaka SHIMADA,^{c,d)} Katsutoshi TERASAWA,^{d)} Fang TANG^{a)}

^{a)}Department of Traditional Chinese Medicine, General Hospital, Tianjin Medical University, China. ^{b)}Department of Kampo Diagnostics, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Japan. ^{c)}Department of Japanese Oriental Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Japan. ^{d)}21st Century COE Program, Toyama Medical and Pharmaceutical University, Japan. (Received April 13, 2004. Accepted June 2, 2004.)

In this study, the effects of Toki-shakuyaku-san on the microcirculation of bulbar conjunctiva in 11 patients with asymptomatic cerebral infarction were investigated with a video-microscopic system. After the administration of Toki-shakuyaku-san for four weeks, the flow volume rates of microcirculatory flow of the bulbar conjunctiva were increased ($p < 0.05$). Hemorheological factors such as whole blood viscosity, plasma viscosity, and erythrocyte deformability were examined. Toki-shakuyaku-san improved whole blood viscosity and erythrocyte deformability ($p < 0.05$), and plasma lipid peroxides decreased. These results suggested that the favorable effects of Toki-shakuyaku-san on cerebrovascular disorders take place via changes in microcirculatory flow, with the mechanisms being considered to be improvements in hemorheological factors and the anti-oxidant effect of Toki-shakuyaku-san.

Key words Toki-shakuyaku-san, microcirculation, asymptomatic cerebral infarction, whole blood viscosity, erythrocyte deformability, anti-oxidant effect.

Introduction

Toki-shakuyaku-san (TSS: Dang-gui-shao-yao-san), one of the famous traditional Chinese prescriptions, has been used to treat obstetric and gynecologic disorders since olden times. TSS is presently also used to treat sterility, anemia at pregnancy, hypopituitarism and menopausal syndrome. Further, TSS was reported to exert an effect against Alzheimer type dementia¹⁾ and cerebrovascular dementia,²⁾ and thus is often used in a clinical setting.

Its mechanisms were reported to affect neuroendocrinology, such as stimulating the synthesis of acetylcholine receptor and increasing the kinds of catecholamine.³⁾ However, there are few reports concerning TSS and brain blood flow and hemorheology clinically. It is reported that asymptomatic cerebral infarction progresses to vascular dementia and cerebral infarction.^{4,5)} A decrease in cerebral blood flow due to endothelial dysfunction from aging and hypertension are thought to be the causative factors.⁶⁾

In this study, we investigated the effect of TSS on the microcirculation and hemorheological factors in patients with asymptomatic cerebral infarction.

Subjects and Methods

Patients. Eleven patients without neurological disorder were diagnosed as asymptomatic cerebral infarction based on high-intensity lesions greater than 3 mm in size on T₂-weighted images coinciding with low-intensity lesions on T₁-weighted on MRI. They visited the Department of

Japanese Oriental (Kampo) Medicine, Toyama Medical and Pharmaceutical University Hospital between October 2001 and March 2002. The group consisted of 5 males and 6 females, aged 50–72 years (mean \pm S.D.). Informed consent was obtained from all patients. Their complications included 5 cases of hypertension, 3 cases of diabetes mellitus and 2 cases of hyperlipidemia. Although some of them were being treated by Western medicines that influenced hemorheological factors, these medicines were not changed from three months before entry into this study until the end of the four-week TSS administration.

Preparation of Toki-shakuyaku-san. TSS was prepared as a hot decoction. It consisted of 6.0g of *Alismatis Rhizoma* (*Alisma orientale* JUZEPCZUK), 5.0g of *Paeoniae Radix* (*Paeonia lactiflora* PALLAS), 4.0g of *Atractylodis Rhizoma* (*Atractylodes lancea* DE CANDOLLE), 4.0g of *Hoelen* (*Poria cocos* WOLF), 3.0g of *Cnidii Rhizoma* (*Cnidium officinale* MAKINO), and 3.0g of *Angelicae Radix* (*Angelica acutiloba* KITAGAWA). These crude drugs were obtained from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan). These pharmacons were boiled in 600ml water for 40 minutes, and 300ml of hot decoction was formed. Patients were orally given the Toki-shakuyaku-san decoction three times a day (300ml/day) for four weeks.

Study protocol. Blood pressure was measured and microcirculation of bulbar conjunctiva was observed by video-microscopic system at about 9:00 a.m. after overnight fasting both just before and right after the four-week period of TSS administration. At the same time, blood was withdrawn from the cubital vein and anti-coagulated in EDTA-2Na (1.5mg/ml) to measure whole blood viscosity, plasma

*To whom correspondence should be addressed. e-mail : hiro510@ms.toyama-mpu.ac.jp

viscosity, erythrocyte deformability, red blood cell (RBC), hemoglobin (Hb), hematocrit (Ht), lipid peroxides (LPO), $\text{NO}_2^-/\text{NO}_3^-$ and fibrinogen.

Measurement of microcirculatory flow. We used a video-microscope system to observe the venules of the bulbar conjunctiva.⁷⁾ The internal diameter (ID) of the vessels (their initial ID was about $18\mu\text{m}$ and they were mostly straight-line) was measured. The traveling distance of one erythrocyte during one second was measured frame by frame and the averaged values were calculated after three estimations as the flow velocity (Fve; $\mu\text{m}/\text{sec}$). Flow volume rate (Fvo; $\mu\text{m}^3/\text{sec}$) was obtained from the equation $\text{Fvo}=(1/2 \text{ ID})^2 \times \pi \times \text{Fve}$.

Measurement of viscosity. The details of blood viscosity measurement were explained in our previous paper.⁸⁾ Whole blood was measured by coneplate rotational viscometer (Bio-rheolizer, Tokyo Keiki Co., Ltd., Tokyo, Japan) at five different points of shear rates (γ) ($19.2, 38.4, 76.4, 192.0, 384.0 \text{ sec}^{-1}$) five times, respectively, and the averages of the five values were calculated. Using the remaining blood samples, this procedure was repeated. Final viscosity was estimated by the average at each point through the average of two repeated tests. Plasma viscosity was estimated at one shear rate (384.0 sec^{-1}) through the average of five values. All viscosity measurements were performed at a constant temperature of 37°C .

Measurement of erythrocyte deformability. The apparatus and sample preparation for measurement of erythrocyte deformability were described in our previous paper.⁹⁾ After high-speed centrifugation, plasma and buffy coat were removed. The remaining packed erythrocytes were washed three times with isotonic phosphate buffer (PBS; $\text{NaCl } 93.4 \text{ mM}, \text{Na}_2\text{HPO}_4 - \text{NaH}_2\text{PO}_4 \text{ } 3.2 \text{ mM}, \text{KCl } 5.0 \text{ mM}, \text{glucose } 5 \text{ mM}, \text{pH } 7.4, 295 \text{ mOsm/kg}$) and resuspended in isotonic PBS to a final concentration of 15%. Erythrocyte deformability was determined by measuring the filtration time required for $400\mu\text{l}$ of 15% red cell suspension to pass through a $5\mu\text{m}$ pore filter (Nucleopore, Costar Co., Ltd., CA, USA) under constant $-10\text{cm H}_2\text{O}$ pressure. Erythrocyte deformability was calculated as the average of six repeated tests.

RBC, Hb, Ht, lipid peroxide and fibrinogen measurement. RBC, Hb and Ht were determined by automatic

counter (Celltac α , MEK-6158, NIHON KOHDEN Co., Tokyo). LPO was measured by the Yagi method,¹⁰⁾ and plasma fibrinogen by the thrombin time method.¹¹⁾

NO measurement. NO is an extremely unstable molecule and rapidly undergoes oxidative degradation to stable inorganic nitrogen oxides $\text{NO}_2^-/\text{NO}_3^-$ that were used here as indices of in vivo NO generation. Serum $\text{NO}_2^-/\text{NO}_3^-$ was measured with an automated system (ENO-10; EICOM Co., Kyoto, Japan), based on the Griess reaction method.

Statistical analysis. Data were presented as mean \pm standard error. Statistical comparisons were made using the Wilcoxon's t-test. The level of statistical significance was defined as $p < 0.05$.

Results

There were no significant differences in blood pressure between before and after the four-week period of TSS administration. As for microcirculatory flow, the mean internal diameter of vessels and flow velocity did not change from before to after the four-week period of TSS administration, but the mean flow volume rate significantly increased from $0.9 \pm 0.09 \times 10^5 \mu\text{m}^3/\text{sec}$ to $1.14 \pm 0.09 \times 10^5 \mu\text{m}^3/\text{sec}$ (Table 1).

Whole blood viscosity was corrected by hematocrit at 45%. There were no significant differences between before and after the four-week period of TSS administration at low shear stress in whole blood viscosity. However, there was significant decrease in the viscosity at high shear stress from $4.14 \pm 0.06 \text{ cp}$ to $4.01 \pm 0.09 \text{ cp}$. Plasma viscosity showed no significant difference at either low or high shear stress. Mean erythrocyte deformability significantly improved from $13.53 \pm 0.35 \text{ msec}$ to $12.91 \pm 0.42 \text{ msec}$ in the four-week period (Table 2). The number of RBC increased from $459.8 \pm 9.3 \times 10^4/\mu\text{l}$ to $473.2 \pm 9.3 \times 10^4/\mu\text{l}$, Hb increased from $13.3 \pm 0.3 \text{ g/dl}$ to $14.1 \pm 0.4 \text{ g/dl}$, and Ht increased from $43.4 \pm 1.0 \%$ to $44.9 \pm 1.0 \%$, all significantly. LPO was significantly decreased from $2.69 \pm 0.32 \text{ nmol/ml}$ before, to $2.18 \pm 0.16 \text{ nmol/ml}$ after the four-week period of TSS administration. $\text{NO}_2^-/\text{NO}_3^-$ and fibrinogen did not change in the four weeks (Table 3).

Table 1 Changes in blood pressure and microcirculatory flow

		Pre-administration	Post-administration
Blood pressure			
Systolic pressure	(mmHg)	132 ± 6	136 ± 5
Diastolic pressure	(mmHg)	81 ± 4	82 ± 4
Microcirculatory flow			
Internal diameter of vessels	(μm)	18.1 ± 0.7	19.2 ± 0.7
Blood flow velocity	($\mu\text{m}/\text{sec}$)	353.3 ± 31.6	389.5 ± 16.2
Blood flow volume	($\times 10^5 \mu\text{m}^3/\text{sec}$)	0.9 ± 0.09	$1.14 \pm 0.09^*$

The results were expressed as mean \pm S.E., $n=11$. Asterisks indicate significant differences from pre-administration (*: $p < 0.05$).

Table 2 Changes in hemorheological factors

		Pre-administration	Post-administration
Corrected whole blood viscosity			
Low shear stress	(cp)	7.02±0.15	6.91±0.28
High shear stress	(cp)	4.14±0.06	4.01±0.09*
Plasma viscosity			
Low shear stress	(cp)	7.52±0.64	7.49±0.83
High shear stress	(cp)	1.52±0.05	1.41±0.03
Erythrocyte deformability	(msec)	13.53±0.35	12.91±0.42*

The results were expressed as mean ± S.E., n=11. Asterisks indicate significant differences from pre-administration (*: $p < 0.05$).

Table 3 Changes in RBC count, plasma lipid peroxides, fibrinogen and $\text{No}_2^-/\text{No}_3^-$

		Pre-administration	Post-administration
Red blood cell	($\times 10^4/\mu\text{l}$)	459.8±9.3	473.2±9.3*
Hemoglobin	(g/dl)	13.3±0.3	14.1±0.4*
Hematocrit	(%)	43.4±1.0	44.9±1.0*
Lipid peroxides	(nmol/l)	2.69±0.32	2.08±0.16*
$\text{No}_2^-/\text{No}_3^-$	(10^{-5}M)	25.0±6.4	25.5±4.8
Fibrinogen	(mg/ml)	184.6±9.5	196.6±11.9

The results were expressed as mean ± S.E., n=11. Asterisks indicate significant differences from pre-administration (*: $p < 0.05$).

Discussion

Asymptomatic cerebral infarction is a significant disease, as it progresses to vascular dementia and cerebral infarction.^{4,5)} The mechanism underlying its cause is considered to be decreasing cerebral blood flow from endothelial dysfunction resulting from both aging and hypertension.⁶⁾ For its treatment, anticoagulants are not commonly used because of the possibility of inducing cerebral bleeding. This means that, under such circumstances, only hypertension receives treatment. In traditional Oriental medicine, there is the concept of "Oketsu", which means blood stasis. There are some prescriptions that are called "anti-Oketsu" drugs for treating blood stasis. TSS is one of these "anti-Oketsu" drugs, and it is used for patients whose health is adversely affected by coldness. It is also often used for gynecologic disease with anemia.

In this study, TSS had no effect on blood pressure, but the blood flow volume of the microcirculation at conjunctive bulbi increased significantly. One of its mechanisms was thought to be the improvement of erythrocyte deformability, and it was supported that the whole blood viscosity at high shear stress and the filtration time of RBC decreased. Further, LPO is an index of radical production, and an increase in production is a contributing factor in arteriosclerosis¹²⁾ and also weakens the NO effect, worsening blood circulation.¹³⁾ In the present study, plasma LPO decreased significantly, and therefore TSS was thought to have a favorable effect on blood circulation.

There was a slight increase in the number of RBC. It has also been reported that TSS had a hematopoietic effect and improved anemia. But as polycythemia is one of the

risk factors of cerebral infarction, TSS is particularly useful for anemic patients.

Until now we have been studying the effects of Kampo formulations such as Keishi-bukuryo-gan¹⁴⁾ and Choto-san.⁸⁾ Comparing their results with the present result, TSS, Keishi-bukuryo-gan and Choto-san all improved microcirculation. TSS and Keishi-bukuryo-gan decreased whole blood viscosity, and TSS and Choto-san improved erythrocyte deformability. Furthermore, Keishi-bukuryo-gan decreased the levels of Ht and fibrinogen, TSS increased the RBC count, and Choto-san decreased the level of total cholesterol. Thus, each formula has its particular features, and they share that of the improvement of the microcirculation.

Generally, the use of Kampo formulas is still determined on the basis of traditional Oriental thinking. However, from the present study, it was possible to select the best formula not only by the method of traditional Oriental thinking but also by taking into account Western medical laboratory data.

Acknowledgments

This study was supported by a Grant-in-Aid for the Funds for Comprehensive Research on Aging and Health from the Japanese Ministry of Health and Welfare. This work was supported by a Grant-in-Aid for the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- 1) Kikuya, T.: Treatment of senile psychic disturbances with ethical

- kampo drugs, applied to health insurance system. (Japanese) *The Journal of Traditional Sino-Japanese Medicine*, **12**, 30-36, 1991.
- 2) Saito, M., Su, G., Yagyū, T. and Saito, N.: Kampo (Japanese herbal) treatment of senile dementia. (Japanese) *The Journal of Traditional Sino-Japanese Medicine*, **14**, 518-524, 1993.
 - 3) Hagino, N.: An overview of Kampo medicine, Toki-shakuyaku-san. *Phytother Res*, **7**, 391-394, 1993.
 - 4) Matsubayashi, K., Shimada, K., Kawamoto, A. and Ozawa, T.: Incidental brain lesions on magnetic resonance imaging and neurobehavioral functions in the apparently healthy elderly. *Stroke* **23**, 175-180, 1992.
 - 5) Kobayashi, S., Okada, K., Koide, H., Bokura, H. and Yamaguchi, S.: Incidence of silent brain infarction as a risk factor for clinical stroke. *Stroke* **28**, 1932-1939, 1997.
 - 6) Kobayashi, S., Okada, K. and Yamashita, K.: Incidence of silent lacunar lesion in normal adults and its relation to cerebral blood flow and risk factors. *Stroke* **22**, 1379-1383, 1991.
 - 7) Terasawa, K., Itoh, T., Morimoto, Y., Hiyama, Y. and Tosa, H.: The characteristics of the microcirculation of bulbar conjunctiva in "Oketsu" syndrome. *J. Med. Pharm. Soc. WAKAN-YAKU*, **5**, 200-205, 1988.
 - 8) Yang, Q., Kita, T., Hikiami, H., Shimada, Y., Itoh, T. and Terasawa, K.: Effects of Choto-san on microcirculation of bulbar conjunctiva and hemorheological factors in patients with asymptomatic cerebral infarction. *J. Trad. Med.* **16**, 135-140, 1999.
 - 9) Hikiami, H., Kohta, K., Sekiya, N., Shimada, Y., Itoh, T. and Terasawa, K.: Erythrocyte deformability in "Oketsu" syndrome and its relations to erythrocyte viscoelasticity. *J. Trad. Med.* **13**, 156-164, 1996.
 - 10) Yagi, K.: A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med* **15**, 212-216, 1976.
 - 11) Paar, D.: Reliability of fibrinogen determination (precision, correctness and normal values). (German) *Blut* **23**, 1-6, 1971.
 - 12) Rubinyi, G.M. and Vanhoutte, P.M.: Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle. *Am. J. Physiol.* **250**, H815-H821, 1986.
 - 13) Grunfeld, S., Hamilton, C.A., Mesaros, S., McClain, S.W., Dominiczak, A.F., Bohr, D.F. and Malinski, T.: Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension* **26**, 854-857, 1995.
 - 14) Itoh, T., Terasawa, K., Kazufumi, K., Shibahara, N., Tosa, H. and Hiyama, Y.: Effects of keishi-bukuryo-gan and Trapidil on the microcirculation in patients with cerebro-spinal vascular disease. *J. Med. Pharm. Soc. WAKAN-YAKU* **10**, 251-259, 1993.

Japanese abstract

当帰芍薬散は陰虚証に用いられる代表的な駆瘀血剤である。今回、無症候性脳梗塞患者に当帰芍薬散を投与し血液レオロジー因子等に及ぼす影響を検討した。対象は富山医科薬科大学附属病院和漢診療科外来通院中で頭部MRI上、無症候性脳梗塞と診断した患者11名(男性5名, 女性6名, 平均年齢59.5±8.2歳)である。方法は、当帰芍薬散投与前と4週後に、ビデオ顕微鏡システムを用い、眼球結膜微小血管を観察し、血管内径、血流速度、血流量を測定した。また、採血により血液粘度、赤血球変形能、過酸化脂質、一酸化窒素代謝物、フィブリノーゲン、赤血球数、Hb、Ht値等を測定した。結果は、当帰芍薬散の4週間の投与により、血圧には変化を認めなかったが、眼球結膜微小血管の血流量が増加し、血液レオロジー因子の全血粘度と赤血球変形能の改善を認めた。また、Hb、Ht値の上昇と過酸化脂質値の低下を認めた。

* 〒930-0194 富山市杉谷 2630

富山医科薬科大学和漢薬研究所漢方診断学部門 後藤博三



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

Koichi Yokoyama^{a,*}, Yutaka Shimada^a, Etsuro Hori^b, Nobuyasu Sekiya^a, Hirozo Goto^c, Iwao Sakakibara^d, Hisao Nishijo^b, Katsutoshi Terasawa^a

^a Department of Japanese Oriental Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan

^b Department of Physiology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan

^c Department of Kampo Diagnostics, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan

^d Pharmacognosy and Medical Resources Laboratory, Tsumura and Co., Amimachi, Ibaraki, Japan

Received 22 November 2002; accepted 15 April 2003

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 NO₂⁻/NO₃⁻ 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. © 2004 Elsevier GmbH. All rights reserved.

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

*Corresponding author. Tel.: +81-76-434-7393; fax: +81-76-434-0366.

E-mail address: yokomac@ms.toyama-mpu.ac.jp (K. Yokoyama).

(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 ($\text{NO}\cdot$) (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 $\text{NO}\cdot$ (Bolanos and Almeida, 1999). In this process, large amounts of superoxide anions ($\text{O}_2^{\cdot-}$) 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 peroxynitrate anion (ONOO^-), from the reaction of $\text{NO}\cdot$ with $\text{O}_2^{\cdot-}$, and further hydroxyl radical ($\text{HO}\cdot$) generation, may be a possible mechanism by which neurotoxicity 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 $\text{O}_2^{\cdot-}$ and $\text{HO}\cdot$ 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 10,000g 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)
<i>Uncariae Uncus Cum Ramulus</i>	Hooks and stem of <i>Uncaria sinensis</i> Haviland	3.0
<i>Aurantii Nobilis pericarpium</i>	Peel of <i>Citrus unshu</i> Markovich	3.0
<i>Pinelliae tuber</i>	Tuber of <i>Pinellia ternata</i> Breitenbach	3.0
<i>Ophiopogonis tuber</i>	Root of <i>Ophiopogon japonicus</i> Ker-Gawler	3.0
<i>Poria</i>	Sclerotium of <i>Poria cocos</i> Wolf	3.0
<i>Ginseng radix</i>	Root of <i>Panax ginseng</i> C.A. Meyer	3.0
<i>Chrysanthemi flos</i>	Flower of <i>Chrysanthemum morifolium</i> Ramatulle	3.0
<i>Saphoshnikoviae radix</i>	Root and rhizome of <i>Saposhnikovia divaricata</i> Schischkin	3.0
<i>Glycyrrhizae radix</i>	Root of <i>Glycyrrhiza uralensis</i> Fisher	1.0
<i>Zingiberis rhizoma</i>	Rhizome of <i>Zingiber officinale</i> Roscoe	1.0
<i>Gypsum Fibrosum</i>	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	5.0

Table 2. Yields and O₂[•] 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 AcONH₄ (pH 3.6) and (B) CH₃CN. 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. 1 and 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 anesthetic 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.

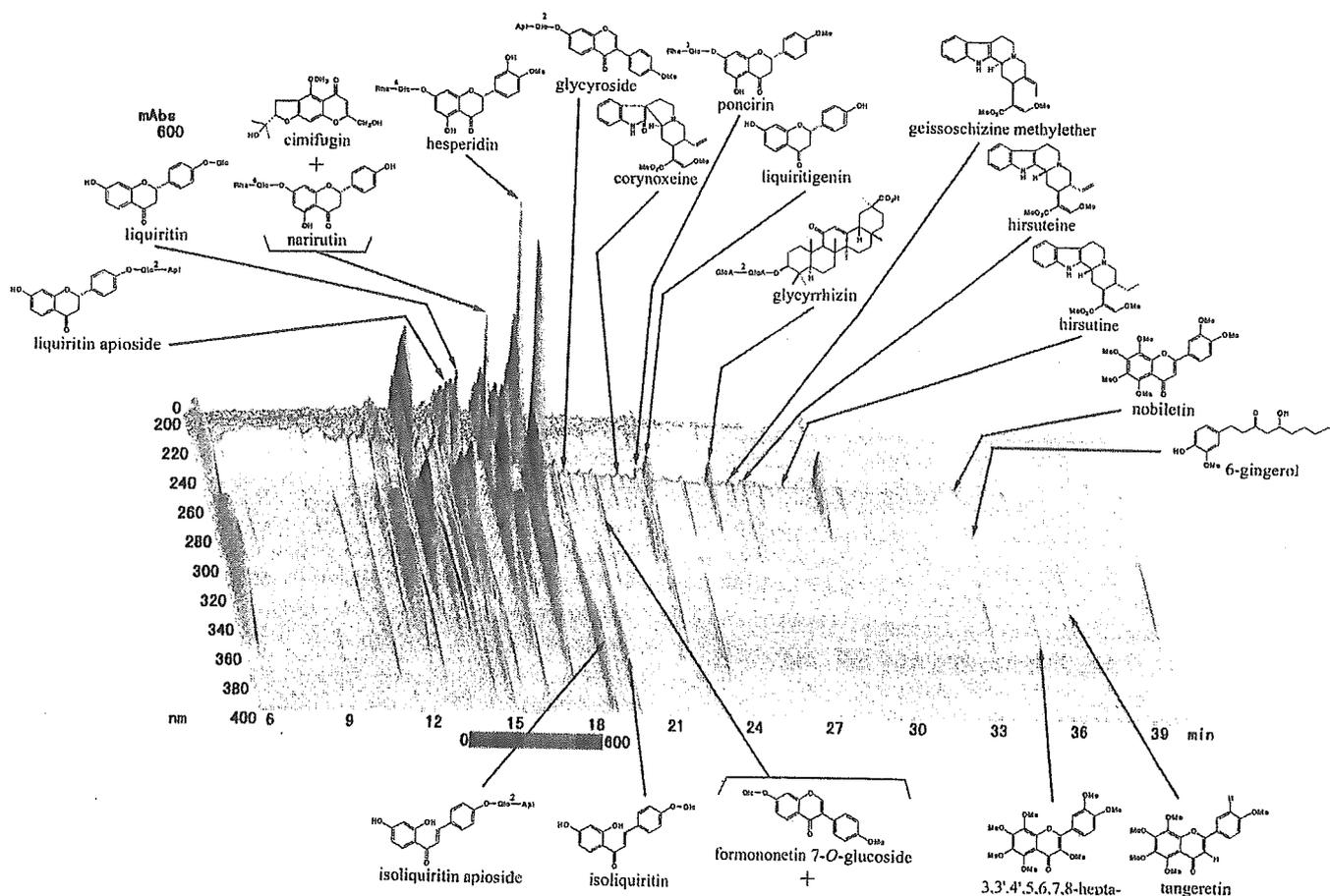


Fig. 1. Three-dimensional HPLC profile of the methanol solution of Choto-san water extract (CSE).

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, NO₂⁻/NO₃⁻ and free radical scavenging activities against both O₂^{-•} 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 NO₂⁻/NO₃⁻

NO₂⁻/NO₃⁻ 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 O₂^{-•} and HO• scavenging activities

Measurement of O₂^{-•} 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), O₂^{-•} and HO• scavenging activities of these samples were assessed by electron spin resonance (ESR) technique (Buettner, 1987).

In this experiment, O₂^{-•} 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

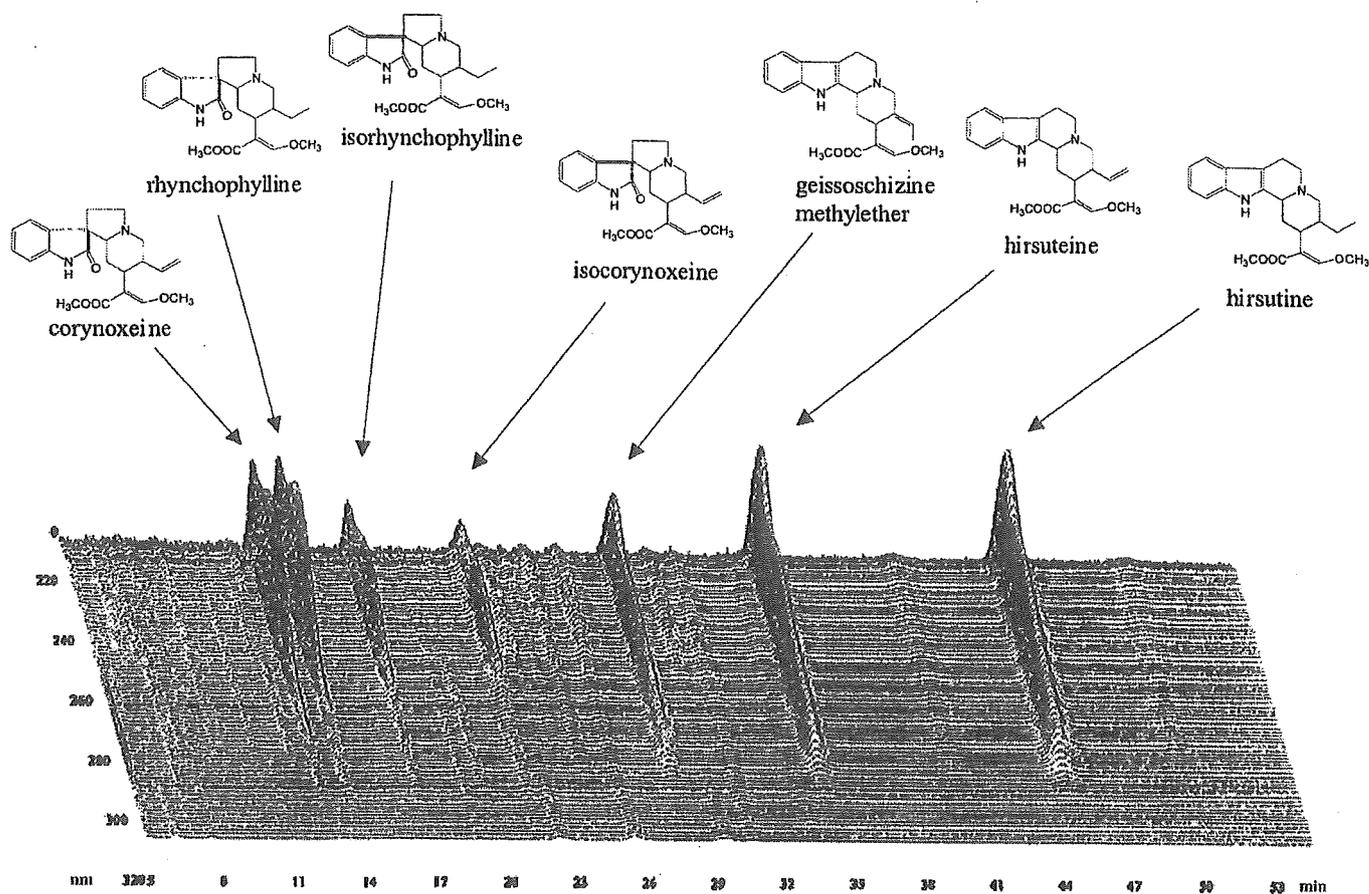


Fig. 2. Three-dimensional HPLC profile of the methanol solution of *U. sinensis* water extract (USE).

50 μ l of 0.4 U/ml XOD (Roche, Indianapolis, USA) and quick mixing, 200 μ l 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:

$$\text{Scv (\%)} = \frac{S/\text{MBLNC} - S/\text{MSAMPLE}}{S/\text{MBLNC}} \times 100,$$

where S/MBLNC 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 \cdot was generated by the Fenton reaction (Kohno et al., 1991) consisting of 75 μ l of 0.1 mM H₂O₂, 50 μ l of each sample, 20 μ l of 92 mM DMPO and 75 μ l of 0.1 mM FeSO₄. The spectrum of DMPO-OH was measured at 1 min after the addition of H₂O₂. 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. O₂ \cdot^- and HO \cdot scavenging activities of each sample (50 μ l) were assessed by ESR technique, and then IC₅₀ (inhibition concentration 50%) was calculated.

Statistical analysis

Values were expressed as mean \pm 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.

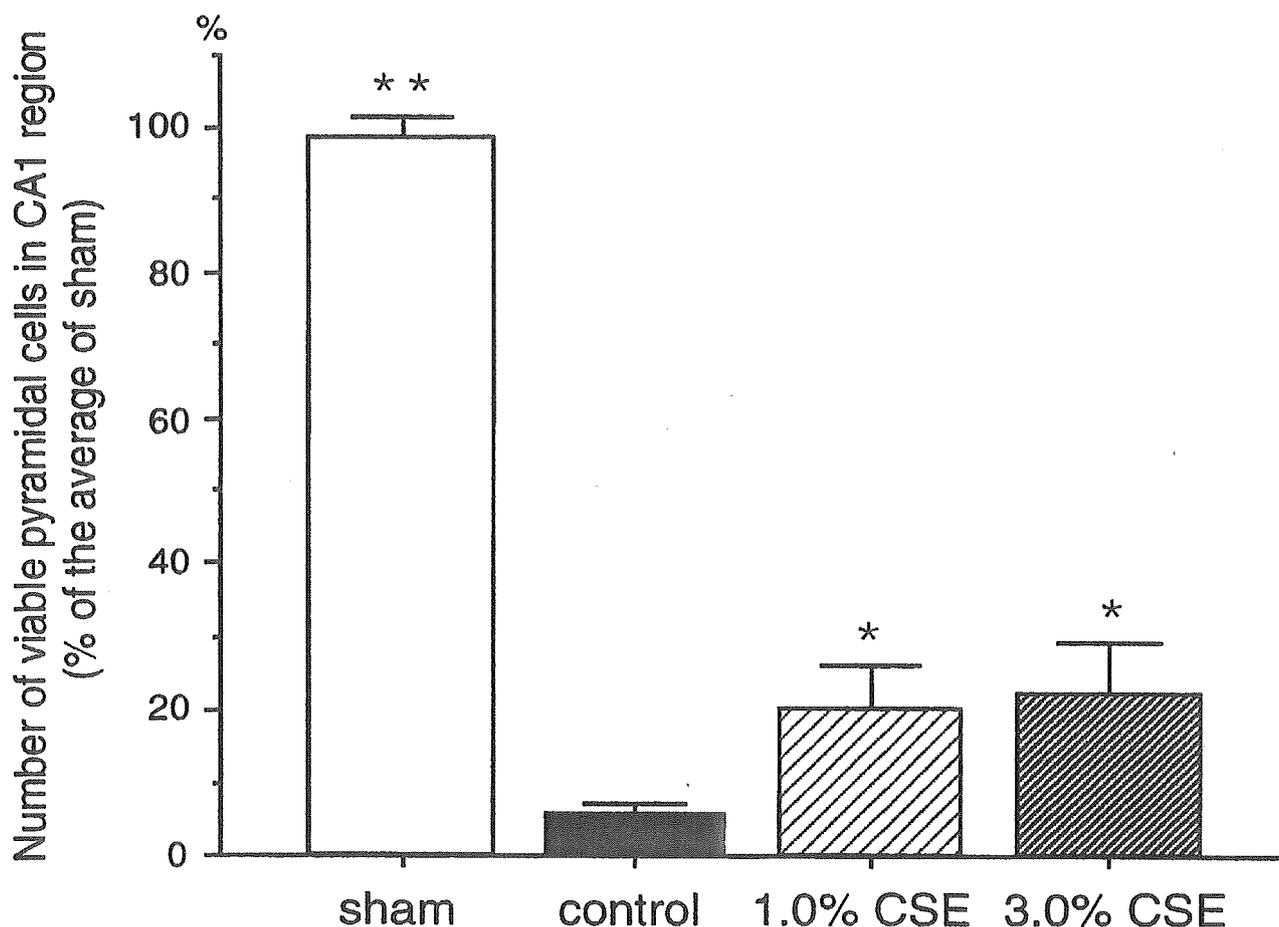


Fig. 3. Numbers of viable pyramidal cells in hippocampal CA1 region at 7 days after i/rp; sham-operated group (sham), i/rp without treatment group (control), i/rp with 1.0% CSE-treated group (1.0% CSE), i/rp with 3.0% CSE-treated group (3.0% CSE). Each column represents the mean \pm S.E. of 10 animals. * 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^\bullet 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^\bullet scavenging activities in the hippocampus.

The O_2^- and HO^\bullet 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^\bullet 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).

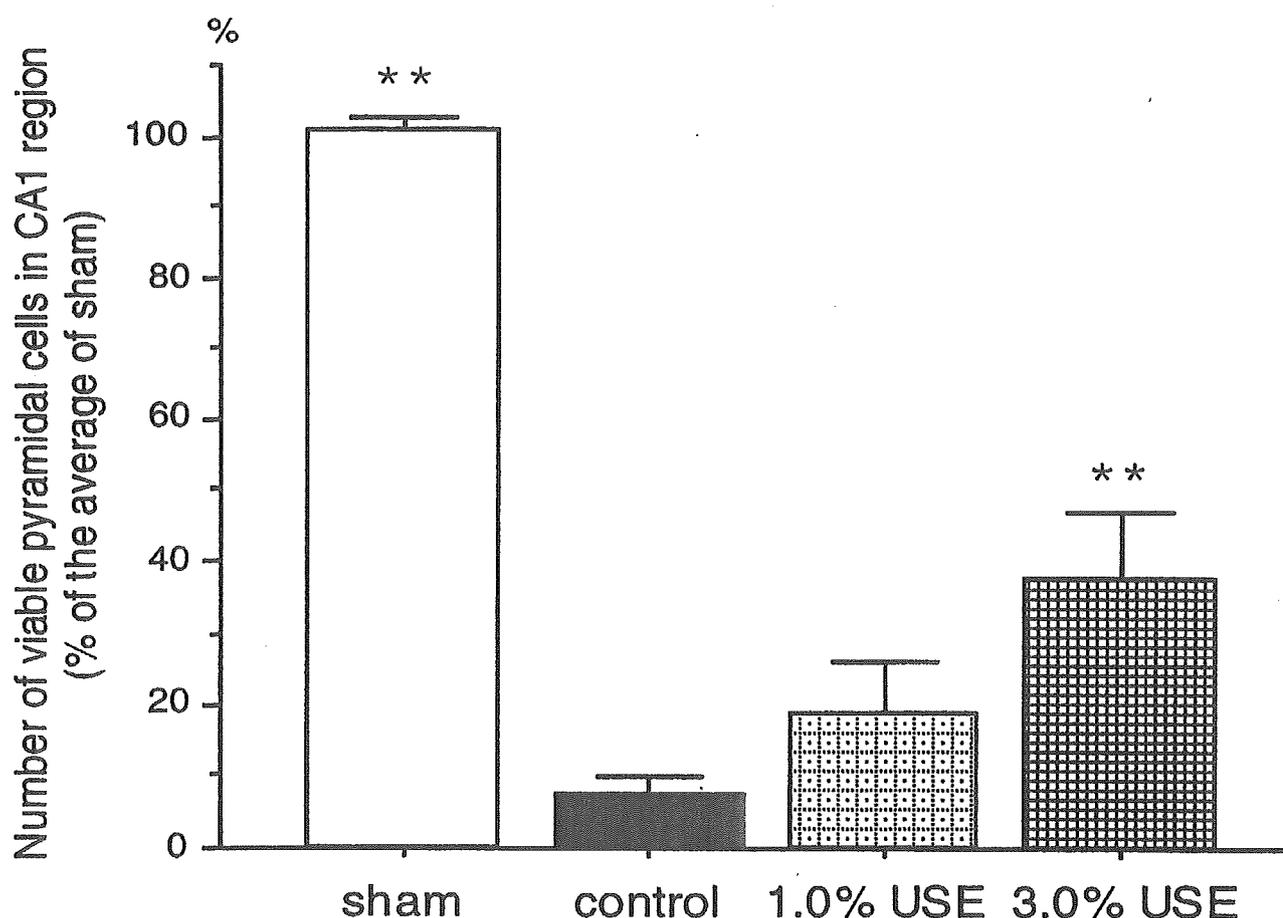


Fig. 4. Numbers of viable pyramidal cells in hippocampal CA1 region at 7 days after i/rp; sham-operated group (sham), i/rp without treatment group (control), i/rp with 1.0% USE-treated group (1.0% USE), i/rp with 3.0% USE-treated group (3.0% USE). Each column represents the mean \pm S.E. of 10 animals.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

O_2^- and HO^\bullet 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^\bullet . Other extracts, especially from *Poria*, *Chrysanthemi flos* and *Zingiberis rhizoma*, have relatively high scavenging activities against O_2^- and HO^\bullet .

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

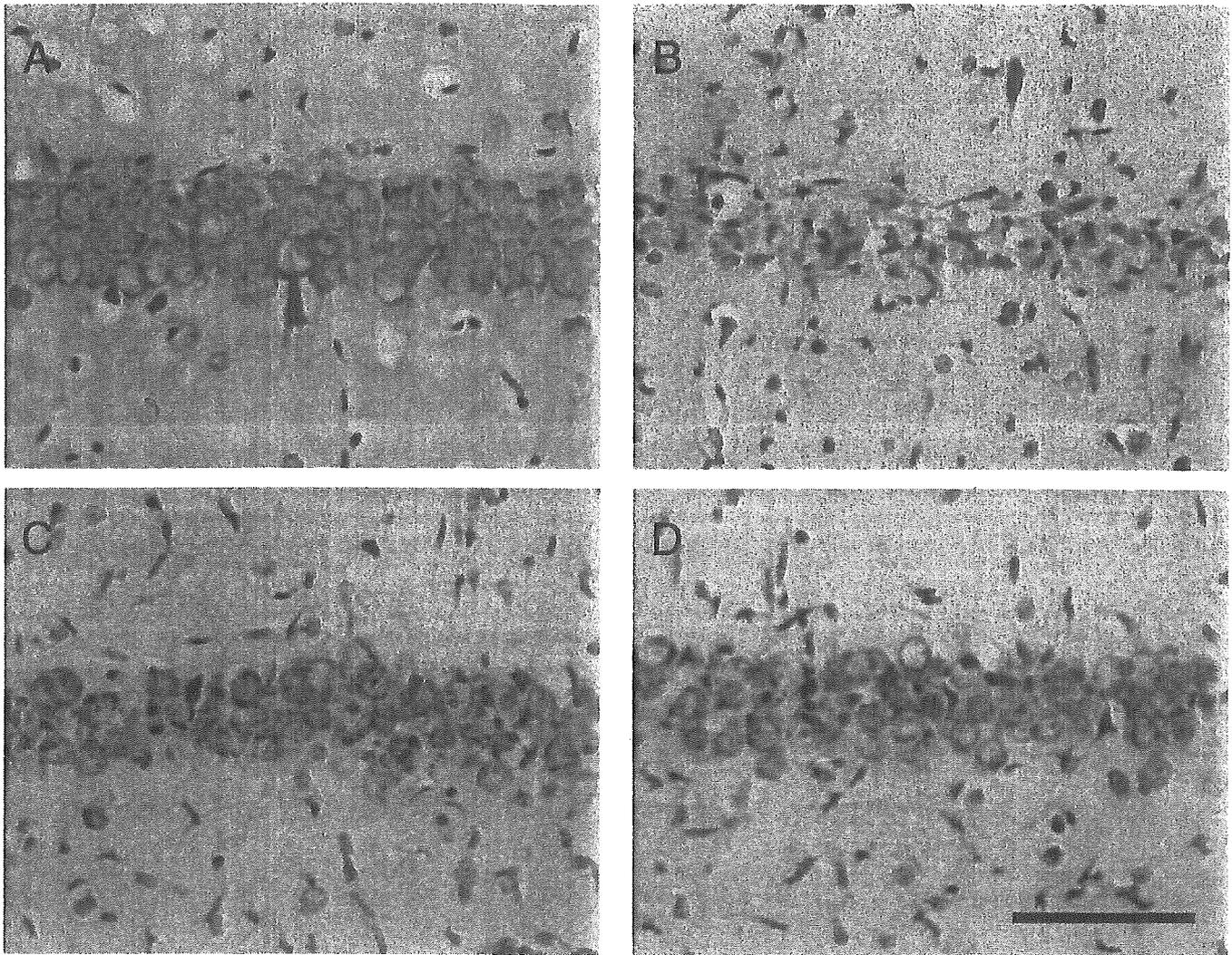


Fig. 5. Protective effect of CSE and USE on delayed neuronal death. Representative photographs of pyramidal cells in hippocampal CA1 region of sham-operated group (A, sham), i/rp without treatment group (B, control), i/rp with 3.0% CSE-treated group (C, 3.0% CSE) and i/rp with 3.0% USE-treated group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

Table 3. O_2^- and HO^\bullet 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 \pm 1.0	42.6 \pm 1.1**	34.6 \pm 2.7**
HO^\bullet scavenging activity (%)	10.8 \pm 3.2	24.4 \pm 1.4**	22.3 \pm 2.7**

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

Table 4. O_2^- and HO^\bullet 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 \pm 2.1	44.1 \pm 1.4**	41.1 \pm 1.3**
HO^\bullet scavenging activity (%)	13.8 \pm 1.5	20.4 \pm 1.0**	23.0 \pm 1.9**

Mean \pm 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

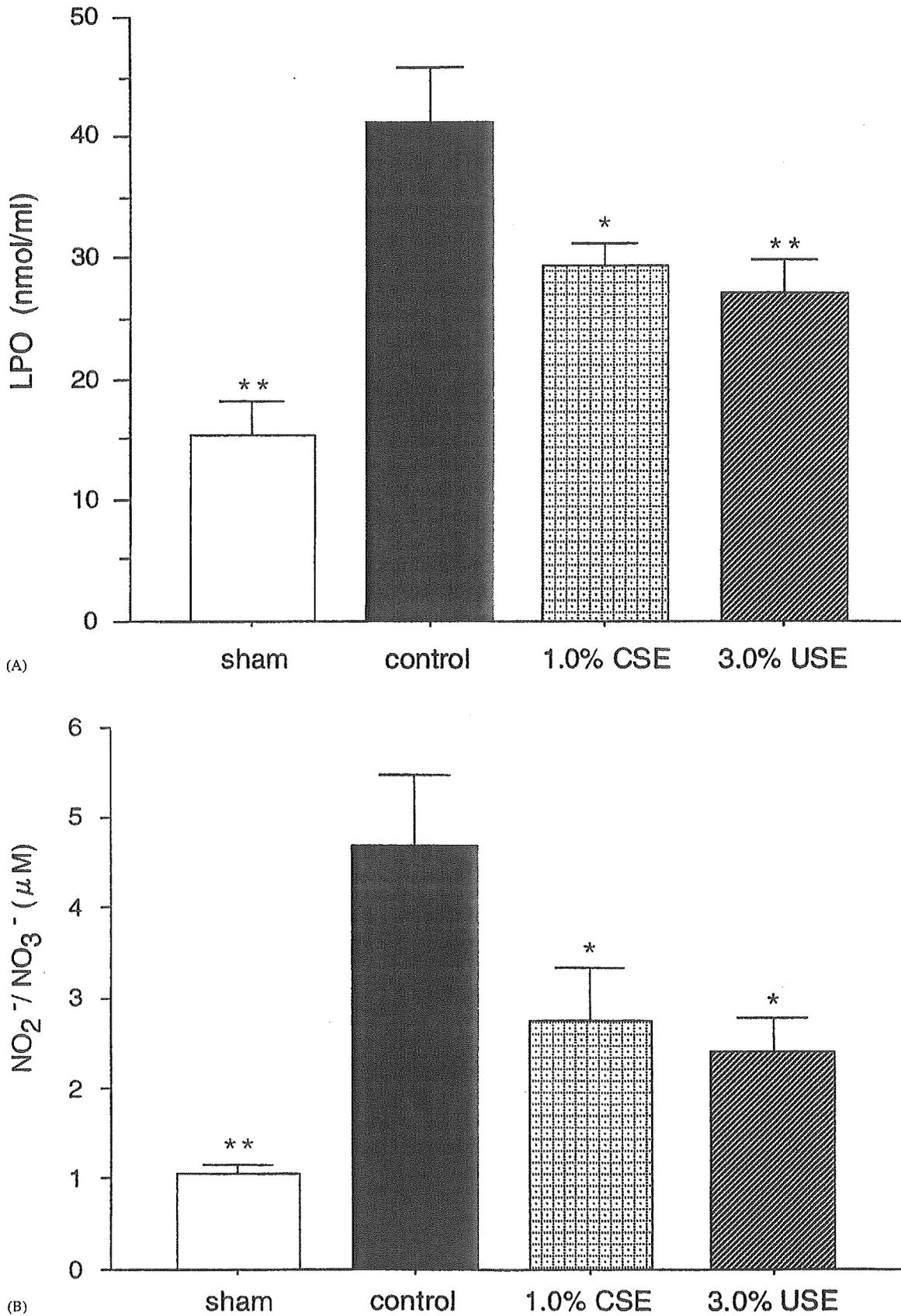


Fig. 6. LPO (A) and NO₂⁻/NO₃⁻ (B) levels in homogenized hippocampus at 48 h after i/rp; sham-operated group (sham), i/rp without treatment group (control), i/rp with 1.0% CSE-treated group (1.0% CSE), i/rp with 3.0% USE-treated group (3.0% USE). Each column represents mean ± S.E. of eight animals. **p* < 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 $O_2^{\cdot-}$ and HO^{\cdot} 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, corynoxine, rhynchophylline, isorhynchophylline and isocorynoxine, and indole alkaloids, geissoschizine methyl ether, hirsuteine and hirsutine (Shimada et al., 1999; Sakakibara et al., 1998). Rhynchophylline, isorhynchophylline, corynoxine, isocorynoxine and hirsutine exhibit vasodilative and Ca^{2+} channel blocking activity in isolated rat thoracic aorta (Horie et al., 1992). Hirsuteine non-competitively 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 [3H] 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 ische-

mia 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-mediated 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 vasoconstriction 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.

Acknowledgements

This work was supported by a Health Sciences Research Grant for Comprehensive Research on Aging and Health from the Japanese Ministry of Health, Labour and Welfare.

References

- Bagenholm, R., Andine, P., Hagberg, H., 1996. Effects of the 21-amino steroid tirilazad mesylate (U-74006F) on brain damage and edema after perinatal hypoxia-ischemia in the rat. *Pediatr., Res.* 40, 399–403.

- Bastianetto, S., Zheng, W.H., Quirion, R., 2000. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br. J. Pharmacol.* 131, 711–720.
- Bolanos, J.P., Almeida, A., 1999. Roles of nitric oxide in brain hypoxia-ischemia. *Biochim. Biophys. Acta* 1411, 415–436.
- Buettner, G.R., 1987. Spin trapping: ESR parameters of spin adduct. *Free Radical Biol. Med.* 3, 259–303.
- Calapai, G., Crupi, A., Firenzuoli, F., Marciano, M.C., Squadrito, F., Inferrera, G., Parisi, A., Rizzo, A., Crisafulli, C., Fiore, A., Caputi, A.P., 2000. Neuroprotective effects of Ginkgo biloba extract in brain ischemia are mediated by inhibition of nitric oxide synthesis. *Life Sci.* 67, 2673–2683.
- Goto, H., Shimada, Y., Tanikawa, K., Sekiya, N., Shintani, T., Terasawa, K., 1999. Effect of *Uncariae Ramulus* et *Uncus* on endothelium in spontaneously hypertensive rats. *Am. J. Chin. Med.* 27, 339–345.
- Goto, H., Sakakibara, I., Shimada, Y., Kasahara, Y., Terasawa, K., 2000. Vasodilator effect of extract prepared from *Uncariae Ramulus* on isolated rat aorta. *Am. J. Chin. Med.* 28, 197–203.
- Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]-nitrate in biological fluids. *Anal. Biochem.* 126, 131–138.
- Hong, J.T., Ryu, S.R., Kim, H.J., Lee, J.K., Lee, S.H., Yun, Y.P., Lee, B.M., Kim, P.Y., 2001. Protective effect of green tea extract on ischemia/reperfusion-induced brain injury in Mongolian gerbils. *Brain Res.* 888, 11–18.
- Horie, S., Yano, S., Aimi, N., Sakai, S., Watanabe, K., 1992. Effects of hirsutine, an antihypertensive indole alkaloid from *Uncaria rhynchophylla*, on intracellular calcium in rat thoracic aorta. *Life Sci.* 50, 491–498.
- Hsieh, C.L., Tang, N.Y., Chiang, S.Y., Hsieh, C.T., Lin, J.G., 1999. Anticonvulsive and free radical scavenging actions of two herbs, *Uncaria rhynchophylla* (Miq) Jack and *Gastrodia elata* Bl., in kainic acid-treated rats. *Life Sci.* 65, 2071–2082.
- Inanami, O., Watanabe, Y., Syuto, B., Nakano, M., Tsuji, M., Kuwabara, M., 1998. Oral administration of (–)catechin protects against ischemia-reperfusion-induced neuronal death in the gerbil. *Free Radical Res.* 29, 359–365.
- Ishimaru, H., Takahashi, A., Ikarashi, Y., Maruyama, Y., 1997. Effects of MK-801 and pentobarbital on cholinergic terminal damage and delayed neuronal death in the ischemic gerbil hippocampus. *Brain Res. Bull.* 43, 81–85.
- Kanatani, H., Kohda, H., Yamasaki, K., Hotta, I., Nakata, Y., Segawa, T., Yamanaka, E., Aimi, N., Sakai, S., 1985. The active principle of the branchlets and hook of *Uncaria sinensis* Oliv. examined with a 5-hydroxytryptamine receptor binding assay. *J. Pharm. Pharmacol.* 37, 401–404.
- Kirino, T., 1982. Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res.* 239, 57–69.
- Kohno, M., Yamada, M., Mitsuta, K., Mizuta, Y., Yoshikawa, T., 1991. Spin-trapping studies on the reaction of iron complexes with peroxides and the effects of water-soluble antioxidants. *Bull. Chem. Soc. Jpn.* 64, 1447–1453.
- Kohno, K., Ohta, S., Kohno, K., Kumon, Y., Mitani, A., Sakaki, S., Kataoka, K., 1996. Nitric oxide synthase inhibitor reduces delayed neuronal death in gerbil hippocampal CA1 neurons after transient global ischemia without reduction of brain temperature or extracellular glutamate concentration. *Brain Res.* 738, 275–280.
- Lafon-Cazal, M., Culcasi, M., Gaven, F., Pietri, S., Bockaert, J., 1993. Nitric oxide, superoxide and peroxynitrite: putative mediators of NMDA-induced cell death in cerebellar granule cells. *Neuropharmacology* 32, 1259–1266.
- Lipton, S.A., Rosenberg, P.A., 1994. Excitatory amino acid as a final common pathway for neurologic disorders. *N. Engl. J. Med.* 330, 613–622.
- Liu, J., Mori, A., 1992. Antioxidant and free radical scavenging activities of *Gastrodia elata* Bl. and *Uncaria rhynchophylla* (Miq.) Jacks. *Neuropharmacology* 31, 1287–1298.
- Mimaki, Y., Toshimizu, N., Yamada, K., Sashida, Y., 1997. Anti-convulsion effects of Choto-san and Chotoko (*Uncariae Uncis* cam *Ramulus*) in mice, and identification of the active principles. *Yakuga. Zasshi* 117, 1011–1021.
- Mitsuta, K., Mizuta, Y., Kohno, M., Hiramatsu, M., Mori, A., 1990. The application of ESR spin-trapping technique to the evaluation of SOD-like activity of biological substances. *Bull. Chem. Soc. Jpn.* 63, 187–191.
- Ohsugi, M., Fan, W., Hase, K., Xiong, Q., Tezuka, Y., Komatsu, K., Namba, T., Saitoh, T., Tazawa, K., Kadota, S., 1999. Active-oxygen scavenging activity of traditional nourishing-tonic herbal medicines and active constituents of *Rhodiola sacra*. *J. Ethnopharmacol.* 67, 111–119.
- O'Neill, M.J., Hicks, C., Ward, M., Panetta, J.A., 1997. Neuroprotective effects of the antioxidant LY231617 and NO synthase inhibitors in global cerebral ischaemia. *Brain Res.* 760, 170–178.
- Plumb, G.W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., Williamson, G., 1998. Antioxidant properties of catechins and proanthocyanidines: effect of polymerization, galloylation and glycosylation. *Free Radical Res.* 29, 351–358.
- Sakakibara, I., Takahashi, H., Terabayashi, S., Yuzurihara, M., Kubo, M., Higuchi, M., Ishige, A., Komatsu, Y., Maruno, M., Okada, M., 1998. Chemical and pharmacological evaluations of Chinese crude drug “Gou-teng”. *Nat. Med.* 52, 353–359.
- Shimada, Y., Terasawa, K., Yamamoto, T., Maruyama, I., Saitoh, Y., Kanaki, E., Takaori, S., 1994. A well-controlled study of Choto-san and placebo in the treatment of vascular dementia. *J. Trad. Med.* 11, 246–255.
- Shimada, Y., Goto, H., Kogure, T., Shibahara, N., Kita, T., Itoh, T., Terasawa, K., 1998. Extract prepared from the hooks and stems of *Uncaria sinensis* prevents glutamate-induced neuronal death in cultured cerebellar granule cells. *J. Trad. Med.* 15, 141–146.
- Shimada, Y., Goto, H., Itoh, T., Sakakibara, I., Kubo, M., Sasaki, H., Terasawa, K., 1999. Evaluation of the protective effects of alkaloids isolated from the hooks and stems of *Uncaria sinensis* on glutamate-induced neuronal death in cultured cerebellar granule cells from rats. *J. Pharm. Pharmacol.* 51, 715–722.
- Shimada, Y., Goto, H., Kogure, T., Shibahara, N., Sakakibara, I., Sasaki, H., Terasawa, K., 2001. Protective effect of phenolic compounds isolated from the hooks and stems of *Uncaria sinensis* on glutamate-induced neuronal death. *Am. J. Chin. Med.* 29, 173–180.

- Shimada, Y., Yokoyama, K., Goto, H., Sakakibara, I., Sekiya, N., Mantani, N., Sakai, S., Terasawa, K., 2002. Protective effect of the hooks and stems of *Uncaria sinensis* against nitric oxide donor-induced neuronal death in cultured cerebellar granule cells. *J. Trad. Med.* 19, 15–20.
- Suk, K., Kim, S.Y., Leem, K., Kim, Y.O., Park, S.Y., Hur, J., Baek, J., Lee, K.J., Zheng, H.Z., Kim, H., 2002. Neuroprotection by methanol extract of *Uncaria rhyncho-phylla* against global cerebral ischemia in rats. *Life Sci.* 70, 2467–2480.
- Terasawa, K., Shimada, Y., Kita, T., Yamamoto, T., Tosa, H., Tanaka, N., Saito, Y., Kanaki, E., Goto, S., Mizushima, N., Fujioka, M., Takase, S., Seki, H., Kimura, I., Ogawa, T., Nakamura, S., Araki, G., Maruyama, I., Maruyama, Y., Takaori, S., 1997. Choto-san in the treatment of vascular dementia: a double-blind, placebo-controlled study. *Phytomedicine* 4, 15–22.
- Watano, T., Nakazawa, K., Obama, T., Mori, M., Inoue, K., Fujimori, K., Takanaka, A., 1993. Non-competitive antagonism by hirsuteine of nicotinic receptor-mediated dopamine release from rat pheochromocytoma cells. *Jpn. J. Pharmacol.* 61, 351–356.
- White, B.C., Sullivan, J.M., DeGracia, D.J., O'Neil, B.J., Neumar, R.W., Grossman, L.I., Rafols, J.A., Krause, G.S., 2000. Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J. Neurol. Sci.* 179, 1–33.



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

Hirozo GOTO,^{a)} Chizuru KIGA,^{b,e)} Takako NAKAGAWA,^{a)} Keiichi KOIZUMI,^{b)} Hiroaki SAKURAI,^{b,c)} Yukari SHIBAGAKI,^{e)} Kazuo OGAWA,^{f)} Naotoshi SHIBAHARA,^{a)} Yutaka SHIMADA,^{c,d)} Ikuo SAIKI^{b,c)}

^{a)}Department of Kampo Diagnostics, Toyama Medical and Pharmaceutical University; ^{b)}Department of Pathogenic Biochemistry, Toyama Medical and Pharmaceutical University; ^{c)}The 21st Century COE Program, Toyama Medical and Pharmaceutical University; ^{d)}Department of Japanese Oriental Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama, 930-0194, Japan; ^{e)}Bioinformatics Division, INTEC Web and Genome Informatics Corporation; 3-23 Shimoshin-Machi, Toyama, 930-0804, Japan; ^{f)}Medical Evaluation Laboratory, Research Division, Tsumura and Co.; 3586 Yoshihara, Ami-machi, Inashiki-gun, Ibaraki, 300-1192, Japan; and ^{g)}Toyama New Industry Organization; 529 Takada, Toyama, 930-0866, Japan. (Received June 14, 2005. Accepted July 11, 2005.)

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-accelerating) 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 A₂-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. NO₂⁻/NO₃⁻ 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 reported²⁾ 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

*To whom correspondence should be addressed. e-mail: hiro510@ms.toyama-mpu.ac.jp

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 °C 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 (*Pernus 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, NaHCO₃ 25.0, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, CaCl₂ 2.5 and glucose 10.0. The solution was maintained at 37°C and bubbled continuously with 5% CO₂ in O₂ 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 A₂ (PLA₂). To examine the effect against thromboxane A₂ - and prostaglandin H₂-induced contraction, PLA₂ (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 NO₂⁻/NO₃⁻, which were used here as indices of in vivo NO generation. Serum NO₂⁻/NO₃⁻ 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 pre-filled with saline. Heparinized whole blood samples were forced to flow through the microchannels under a pressure difference of 20 cm H₂O. 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°C and 25°C. 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°C 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 pre-washed 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 \pm 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 \pm 11.4	333 \pm 11.2	332 \pm 7.2
Blood sugar	(mg/dl)	736 \pm 32.0	590 \pm 74.5	655 \pm 44.0
Triglyceride	(mg/dl)	202 \pm 38.1	163 \pm 25.9	155 \pm 30.4
Lipid peroxide	($\times 10^{-6}$ M)	5.0 \pm 2.8	6.1 \pm 1.6	6.2 \pm 2.8
Fibrinogen	(mg/dl)	287 \pm 34.3	278 \pm 29.6	265 \pm 21.8
Serum No ₂ ⁻ /No ₃ ⁻	($\times 10^{-5}$ M)	20.3 \pm 2.21	9.8 \pm 1.15 ^a	7.7 \pm 0.81 ^a
Microchannel transit time of whole blood	(sec)	69.0 \pm 1.0	65.6 \pm 2.0	64.5 \pm 1.9 ^b

Each value is mean \pm 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 $45.9 \pm 1.1\%$, $59.7 \pm 1.3\%$ and $52.0 \pm 1.2\%$ in control, keishibukuryogan and tokishakuyakusan groups, respectively (mean \pm 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 $37.3 \pm 3.2\%$, $28.6 \pm 3.5\%$ and $25.4 \pm 3.8\%$ in the control, keishibukuryogan and tokishakuyakusan groups, respectively (Fig. 2). PLA₂-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 PLA₂ were $39.0 \pm 3.8\%$, $27.5 \pm 2.4\%$ and $27.7 \pm 1.4\%$ 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 $28.6 \pm 2.6\%$, $20.8 \pm 2.8\%$ and $20.1 \pm 2.3\%$ 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. NO₂⁻/NO₃⁻ 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

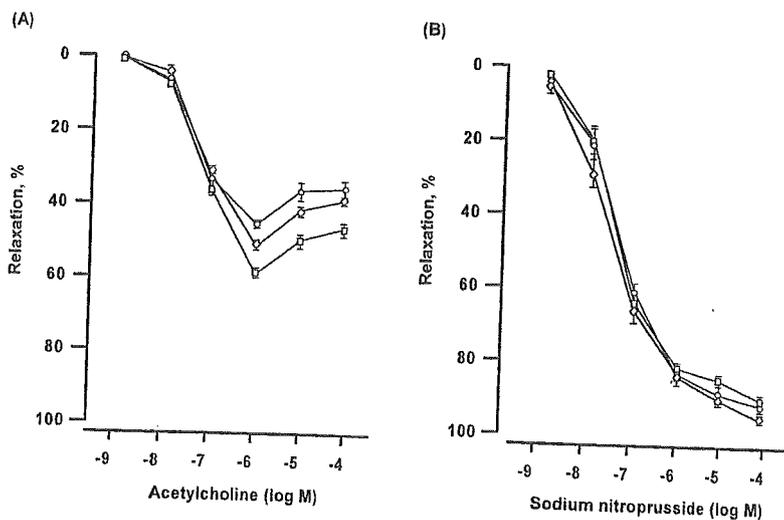


Figure 1 Graph showing A) endothelium-dependent relaxation in response to acetylcholine, B) endothelium-independent relaxation in response to sodium nitroprusside in the aorta of WBN/Kob rats treated for 25 weeks. WBN/Kob control group (○), keishibukuryogan group (□), and tokishakuyakusan group (◇).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

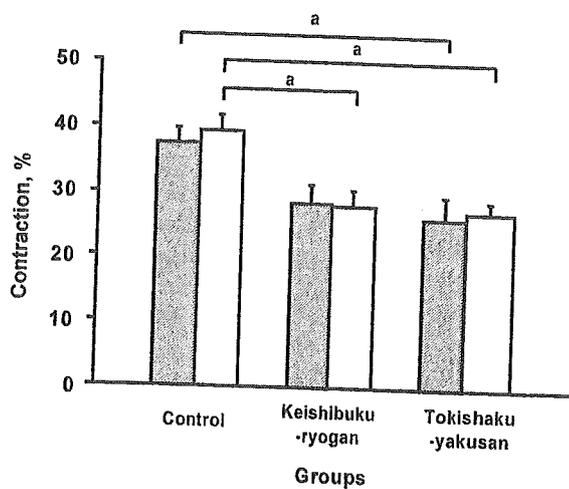


Figure 2 Vasoconstriction treatment on xanthine oxidase (10 mU/ml) in the presence of xanthine (10^{-4} M) (■) and phospholipase (1 U/ml) (□) in the aorta of WBN/Kob rats treated for 25 weeks. All aortas had intact endothelium and had been treated with L-NAME. Contraction was expressed as a percentage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

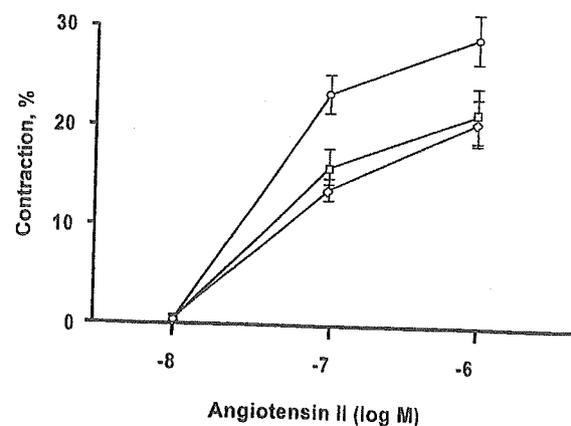


Figure 3 Graph showing angiotensin II induced-contraction in aorta of WBN/Kob rats treated for 25 weeks. WBN/Kob control group (○), keishibukuryogan group (□), and tokishakuyakusan group (◇). Contraction was expressed as the percentage of 60 mM KCl maximum contraction. Shown is the mean \pm S.E. of seven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