Effects of traditional Japanese (Kampo) medicines (orenegedokuto, goreisan and shichimotsukokato) on the onset of stroke and expression patterns of plasma proteins in spontaneously hypertensive stroke-prone rats

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We investigated the effects of three traditional Japanese medicines (TJMs), orenegedokuto, goreisan and shichimotsukokato on the onset of stroke and survival ratio in stroke-prone spontaneously hypertensive rats (SHRSP), and examined the expression patterns of plasma proteins before and after the onset of stroke. Thirty-two 7-week-old male SHRSP were randomly assigned to four groups. The control group received distilled water. Rats in the orenegedokuto, goreisan and shichimotsukokato groups received distilled water containing 0.3% (wt/wt) orenegedokuto, goreisan and shichimotsukokato extracts, respectively, from 9 to 20 weeks old. Blood pressure was measured at 12 and at 14 weeks old. Body weight was measured and blood samples were obtained weekly. Plasma samples were analyzed by ProteinChip technology. As for body weight loss after stroke onset, mean body weight in the control group decreased after 14 weeks old, but those in the orenegedokuto, goreisan and shichimotsukokato groups showed no decrease at 20 weeks old. Systolic blood pressure showed no significant differences among the four groups at 14 weeks old. The survival ratios of the orenegedokuto, goreisan and shichimotsukokato groups were significantly enhanced compared to the control group. Analysis of plasma proteins showed changes in 15 peaks between before and after the onset of stroke in the 3,000-30,000 Da mass ranges in the control group. Especially the peaks at m/z 9,330, 9,480 and 9,700 remarkably decreased after the onset of stroke in the control group. These peaks were identified by western blot analysis as haptoglobin. Interestingly, the decrease was partially prevented by the administration of three TJMs. These results suggest that orenegedokuto, goreisan and shichimotsukokato suppress the onset of stroke in SHRSP independently from the mechanism of an anti-hypertensive effect. At the same time, TJMs affect the expression of proteins associated with the onset of stroke in SHRSP.

Key words orenegedokuto, goreisan, shichimotsukokato, stroke-prone spontaneously hypertensive rat (SHRSP), ProteinChip technology.

Introduction

The stroke-prone spontaneously hypertensive rat (SHRSP) is an experimental model of hypertension in which the animal develops severe cerebral, cardiac and renal lesions and dies from stroke.1 As the cause of stroke, some chromosome areas consisting of several genetic loci related with stroke, are reported.2,3 But the mechanisms of stroke in SHRSP have not yet been clarified. In terms of treatment, anti-hypertensive drugs4 and vitamin E5 are reported to suppress the onset of stroke in SHRSP. In traditional Japanese (Kampo) medicines (TJMs), orenegedokuto,6 goreisan7 and shichimotsukokato8 are reported to suppress the onset of stroke in SHRSP. Their mechanisms are reported to be connected with hypotensive or antioxidative effects, but their details are unclear. Recently, in order to evaluate the effects of TJMs, which are generally prepared from the combination of several crude drugs, an inclusive analytical method such as ProteinChip technology, has been used for profiling the biological mixtures and identifying multiple biomarkers. In our previous study, we reported that hachimijogahogedokuto, goreisan and shichimotsukokato affected the expression patterns of proteins in spontaneously diabetic rats,9 and that keishibukuryogan and tokishaku-yakusus had affected vasofunction with various expression patterns of plasma proteins in spontaneously diabetic rats.10

In the present study, we investigated the effects of orenegedokuto, goreisan and shichimotsukokato on the onset of stroke and survival ratio in SHRSP. We also examined the expression patterns of plasma proteins longitudinally and studied whether they, showing changes between before and after stroke onset, were affected by these TJMs. Furthermore we report that haptoglobin, one of acute-phase proteins induced by infection, tissue injury and malignancy, changes by onset of stroke.

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Materials and Methods

Animals and treatments. Thirty-two 7-week-old male SHRSP were purchased from Japan SLC (Hamamatsu, Japan) and kept in an automatically controlled room (temperature about 23°C and humidity about 60%) with a conventional dark/light cycle. SHRSP were randomly assigned to four groups (control, orangedekuto, goreisan and shchimotsukokato groups). The control group received distilled water. Animals in the orangedekuto, goreisan and shchimotsukokato groups received distilled water containing 0.3% (wt/wt) orangedekuto, goreisan and shchimotsukokato extracts, respectively, from 9 to 20 weeks old. The drug doses used in this study corresponded about ten times the clinical doses. Body weights were also measured every week from 9 to 20 weeks of age. At 12 and 14 weeks old, blood pressure was measured by the tail-cuff method (MK2000, Muromachi Kikai, Tokyo, Japan). Blood samples were obtained from tail vein as plasma every week from 9 to 16 weeks old.

All experiments were conducted in accordance with the Guiding Principles for Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan and had the approval of the Institutional Animal Use and Care Committee of University of Toyama.

Drugs. The pure extracts of orangedekuto, goreisan and shchimotsukokato were purchased from Tsumura & Co. (Tokyo, Japan). The components of these TJMs are summarized in Figure 1. Aqueous extracts were made from these crude drugs and were spray dried to obtain powder. HPLC profiles of orangedekuto, goreisan and shchimotsukokato extracts are shown in Figure 2, 3 and 4, respectively.

Plasma sample preparation for SELDI protein profiling. Plasma samples were centrifuged at 3,000 rpm for 10 min to remove insoluble debris and stored at -80°C until used in the surface enhanced laser desorption/ionization (SELDI) profiling study. Samples were thawed and diluted at a ratio of 1:10 (v/v) in urea denaturing buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, 2% ampholyte) and incubated for 20 min on ice.

SELDI protein profiling. The ProteinChip Arrays were assembled into a deep-well type Bioprocessor assembly (Ciphergen Biosystems, Fremont, CA) and then equipped in Laboratory Automation Workstation Biomek® 2000 (Beckman Coulter, Fullerton, CA). Prior to sample loading, CM10 (cation exchange) arrays were equilibrated with 150 μl of buffer (100 mM sodium acetate, pH 4.0) into each well and then pre-washed two times for 5 min on a shaker at room temperature. The arrays were added with 90 μl of buffer and 10 μl of diluted plasma sample into each well, incubated for 30 min on a shaker at room temperature, and then washed three times with 150 μl of buffer for 5 min on a shaker at room temperature. After rinsing twice with 200 μl of deionized water, the arrays were removed from the Bioprocessor assembly and air-dried. One μl of 50% solution of the energy-adsorbing molecule (EAM); sinapinic acid (SPA) (Ciphergen Biosystems) in 50% (v/v) acetonitrile, 0.5% (v/v) trifluoroacetic acid, was applied twice onto each ProteinChip Array, letting the array surface air-dry between the two SPA applications.

The ProteinChip Arrays were analyzed using a ProteinChip Biology System Reader (Model PBS-Ii; Ciphergen Biosystems). The protein masses were calibrated externally using purified peptide and protein standards (Ciphergen Biosystems). The m/z range of 3,000-30,000 was selected for analysis because it contained the majority of the resolved proteins/peptides. The m/z range of 0-3,000 was eliminated from analysis because this area contains adducts of EAM and possibly other chemical contaminants.

SDS-PAGE and western blot analysis. Plasma proteins

<table>
<thead>
<tr>
<th>Formulas</th>
<th>Crude Drugs (Japanese : Scientific name)</th>
<th>g</th>
</tr>
</thead>
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<tr>
<td>Shichimotsukokato</td>
<td>Paeoniae Radix (芍薬: Paeonia lactiflora PALLAS)</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Angelicae Radix (当帰: Angelica acutiloba KITAGAWA)</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Astragali Radix (黄耆: Astragalis membranaceus BUNGE)</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Rehmanniae Radix (地黄: Rehmannia glutinosa LIBOSCHITZ)</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Cnidii Rhizoma (川芎: Cnidium officinale MAKINO)</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Uncariae Uncis Cum Ramulus (釣藤鉤: Uncaria rhynchophylla MIQUEL)</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Phellodendri Cortex (黄柏: Phellodendron amurense RUPRECHT)</td>
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Fig. 1 Composition of orangedekuto, goreisan and shchimotsukokato
Fig. 2 Three-dimensional HPLC profile of orengedokuto

Fig. 3 Three-dimensional HPLC profile of gorean
were separated under reducing conditions on 12.5% SDS-PAGE gels (9 cm × 9 cm) and transferred onto a PVDF membrane (Millipore, Tokyo, Japan). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd., Suita, Japan) overnight at 4°C. Primary rabbit anti-rat haptoglobin antibody (Life Diagnostics, Inc., West Chester, PA) and secondary HRP-conjugated anti-rabbit IgG were diluted 1:1000, and visualized with the ECL system (Amersham Biosciences, UK).

**Data analysis.** 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 the supposed proteins/peptides in the samples, baselines were subtracted and the intensities normalized on the software. 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. We used the Mann-Whitney U test for nonparametric data sets to compare the peak intensities of the protein profiling results among different groups. Survival ratios were analyzed for differences by the Kaplan-Meier method followed by the long-rank test. A p value <0.05 was considered statistically significant.

**Results**

The changes in mean body weight in every group, except rats that died, are shown in Figure 5A. In the control group, mean body weight decreased from 14 weeks old, indicating the onset of stroke. In the orangedokuto and shichimotsukakato groups, mean body weights did not decrease until the age of 20 weeks. In the goreisan group, mean body weight decreased from 14 to 16 weeks old, but it recovered from 18 to 20 weeks old. The survival ratios of the orangedokuto, goreisan and shichimotsukakato groups were significantly higher than that of the control group (Fig.5B).

Systolic blood pressure in the goreisan and shichimotsukakato groups was significantly lower than in the control group at 12 weeks old (p<0.05), but at 14 weeks old there were no significant differences among the four groups (Fig.6).

Spectral analysis of samples was performed in duplicate using the ProteinChip software program. Approximately 195 peaks per spectrum were detected in the 3,000-30,000 Da mass range. Fifteen peaks changed between before and after the onset of stroke in the control group. Especially, the three peaks at m/z 9,330, 9,480 and 9,700 decreased remarkably after stroke onset in the control group. But in the three groups treated with TJM, the decreases of these peaks were delayed compared to those in the control group.
Fig. 5 A) Changes of body weight in four SHRSP groups. Control group (●, n=8), orangedokuto group (○, n=8), goeisan group (▲, n=8) and shichimotsukokato group (□, n=8). Values of changes in body weight from 9 weeks old are expressed as mean±S.D.
B) Kaplan-Meier survival curves of four SHRSP groups. Control group (●), orangedokuto group (○), goeisan group (▲) and shichimotsukokato group (□); *p<0.05, **p<0.01 vs. control group.

Fig. 6 Effects of orangedokuto, goeisan and shichimotsukokato on systolic blood pressure in SHRSP. Open columns are 12 weeks old and closed columns are 14 weeks old. Each column represents mean±S.D. (n=8); *p<0.05 vs. the control group.

The fifteen peaks that changed between before and after the onset of stroke in the control group, the averages of peak intensities, and a comparison of these peaks at 16 weeks old in the four groups are summarized in Table 1. The changes in peaks were similar in the three groups treated with TJMs. The peaks at m/z 7,932, 15,069, 15,179, 15,375 and 15,390 decreased or tended to decrease, and the peaks at m/z 14,637, 27,549 and 27,751 increased or tended to increase compared to the control group.

The peaks at m/z 9,330, 9,480 and 9,700 were identified by the standard LC-MS/MS method as haptoglobin (Hp).\(^{11}\) In the present study, western blot analysis using a polyclonal antibody to Hp was performed (Fig. 8). The antibody recognized two proteins with molecular weight of approximately 10 and 40 kDa, which corresponded to Hpα and Hpβ chains, respectively. Expression of Hpα and Hpβ in plasma was comparable in 9-week-old control and TJM-treated rats. However, Hp expression was significantly decreased in 16-week-old SHRSP. Interestingly, the decrease was partially prevented by the administration of TJMs, and especially in the shichimotsukokato group.

**Discussion**

SHRSP is an experimental model of stroke and one of its causes was reported to be endothelial dysfunction based on vascular necrosis due to decreased brain circulation and hypertension.\(^{12}\) It was reported, on the basis of chromosomal mapping, that several genetic loci were related to
Fig. 7 Changes of peaks at m/z 9,330, 9,480, and 9,700 peaks from 9 to 16 weeks old in four SHRSP groups. Blank columns have no data because of haemolytic samples. Maximum relative peak intensity was 25 and m/z range was 9,200-9,800. Black-edged rectangles represent maximum body weight before weight loss due to stroke. Gray squares are the points at which body weight decreased more than 10 g compared to that one week earlier.

Table 1. Summary of m/z values of plasma proteins expressions that changed between before and after the onset of stroke in the control group, and comparison of the four SHRSP groups at 16 weeks old.

<table>
<thead>
<tr>
<th>m/z</th>
<th>SHRSP</th>
<th>Orengedokuto</th>
<th>Goreisan</th>
<th>Shichimotsukokato</th>
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<tbody>
<tr>
<td>4665</td>
<td>0.241 ± 0.076</td>
<td>0.990 ± 1.783</td>
<td>0.163 ± 0.132</td>
<td>1.000 ± 1.495</td>
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<tr>
<td>4740</td>
<td>0.174 ± 0.081</td>
<td>0.343 ± 0.245</td>
<td>0.187 ± 0.124</td>
<td>0.342 ± 0.269</td>
</tr>
<tr>
<td>4850</td>
<td>0.083 ± 0.062</td>
<td>0.283 ± 0.365</td>
<td>0.198 ± 0.138</td>
<td>0.437 ± 0.456</td>
</tr>
<tr>
<td>7932</td>
<td>6.436 ± 4.732</td>
<td>3.064 ± 2.477</td>
<td>2.130 ± 0.788</td>
<td>2.116 ± 0.733</td>
</tr>
<tr>
<td>9330</td>
<td>0.091 ± 0.078</td>
<td>1.697 ± 4.151</td>
<td>0.143 ± 0.070</td>
<td>2.581 ± 4.308</td>
</tr>
<tr>
<td>9480</td>
<td>0.171 ± 0.071</td>
<td>1.366 ± 3.061</td>
<td>0.246 ± 0.082</td>
<td>3.637 ± 5.836</td>
</tr>
<tr>
<td>9700</td>
<td>0.148 ± 0.063</td>
<td>0.405 ± 0.570</td>
<td>0.204 ± 0.086</td>
<td>0.845 ± 1.205</td>
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<tr>
<td>14637</td>
<td>0.762 ± 0.466</td>
<td>1.210 ± 0.405</td>
<td>1.116 ± 0.287</td>
<td>1.241 ± 0.159</td>
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<tr>
<td>15069</td>
<td>2.385 ± 0.473</td>
<td>1.409 ± 0.521</td>
<td>1.388 ± 0.344</td>
<td>1.331 ± 0.763</td>
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<tr>
<td>15375</td>
<td>4.453 ± 3.600</td>
<td>1.720 ± 1.539</td>
<td>1.260 ± 0.648</td>
<td>1.341 ± 0.604</td>
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<tr>
<td>15390</td>
<td>4.743 ± 3.586</td>
<td>2.260 ± 2.088</td>
<td>1.410 ± 0.536</td>
<td>1.687 ± 0.657</td>
</tr>
<tr>
<td>27549</td>
<td>1.922 ± 0.812</td>
<td>2.956 ± 0.913</td>
<td>3.230 ± 0.505</td>
<td>3.514 ± 0.646</td>
</tr>
<tr>
<td>27751</td>
<td>0.901 ± 0.416</td>
<td>1.406 ± 0.431</td>
<td>1.524 ± 0.222</td>
<td>1.670 ± 0.269</td>
</tr>
</tbody>
</table>

Underline: p<0.05 vs, control by Mann-Whitney U test.
stroke, and they were independent of hypertension.\textsuperscript{2,3} But
the main gene has not been identified and the cause of
stroke is unclear. It has been shown that calcium antagonists
are useful against the onset of stroke in SHRSP, and that
they have a neuroprotective effect at a dose with hypotensive
effect.\textsuperscript{4,5} Furthermore, vitamin E is also reported to suppress
the onset of stroke in SHRSP. Its inhibitory mechanisms are
considered to be anti-hypertensive and anti-coagulative
properties, based on the antioxidative effect of vitamin E.\textsuperscript{5,6}

Orendokeduto, gorenisan and shichimotsukokato, used
in the present study, have already been reported to be effective
for inhibiting stroke in SHRSP. Orendokeduto is reported
to have a hypotensive effect.\textsuperscript{5,6} Goreisan and its
related formulations are reported to suppress stroke without
decreasing blood pressure and its regulatory mechanism is
thought to normalize body water distribution.\textsuperscript{7} Shichimotsuku-
kato has also shown to suppress stroke without decreasing
blood pressure, through the mechanism considered to be
scavenging $\mathrm{O}_2^-$ and inhibiting $\mathrm{O}_2^-$ production.\textsuperscript{5,6} In these
reports, the means of blood pressure of SHRSP were different,
and further study will be needed to study hypotensive ef-
fects of TJMs on SHRSP. In this study, these three TJMs
apparently suppressed the onset of stroke and prolonged the
life span of SHRSP. As blood pressure was not significantly
different among the four groups at 14 weeks old, these ef-
fects are thought not to be related to the decrease of blood
pressure. There was no common crude drug among these
three formulas, and only Phellodendria Cortex was con-
tained in both orendokeduto and shichimotsukokato (Fig. 1).
So it is difficult to suppose that a specific crude drug
worked to suppress the onset of stroke and to prolong the
life span of SHRSP. Therefore, in order to further evaluate
the influence of TJMs on the onset of stroke, a comparison
of the expression patterns of plasma proteins was attempted.
We previously reported that hachimijiogan, keishibukuryogun
and tokishakuyakusan affected the expression patterns of
plasma proteins in spontaneously diabetic rats with nephropathy.\textsuperscript{9,10} In those studies, the changes in the expres-
sion patterns of plasma proteins were different with each
formula. However, present study shown that some proteins
had similar expression changes in spite of the use of different
formulations. In the fifteen protein peaks changing be-
tween before and after the onset of stroke in the control
group, several peaks were scattered and there were no sig-
ificant differences statistically. But seven peaks, at $m/z$
4,850, 9,330, 9,480, 9,700, 14,637, 27,549 and 27,751, in-
creased similarly in the orendokeduto, gorenisan and
shichimotsukokato groups, and five peaks, at $m/z$ 7,932,
15,069, 15,179, 15,375 and 15,390, contrarily decreased in the three groups. As for the fifteen protein peaks that changed between before and after the onset of stroke in the control group in this study, the same changes in certain peaks were observed in spite of the use of different TJMs.

Three peaks, at m/z 9,330, 9,480 and 9,700, decreased remarkably after stroke onset in the control group. They were clearly identified as Hp. Inflammation-sensitive plasma proteins like Hp have already been reported to be related to stroke. But the cause of decreasing Hp after the onset of stroke in this study remains unclear. Hp is known to bind free haemoglobin released from senescent erythrocytes or during episodes of haemolysis. But it is difficult to imagine that the small cerebral bleeding in SHRSP is enough to decrease Hp in plasma. On the other hand, the genotype of Hp is reported to predict the effect of antioxidative protection. In addition, Hp is thought to be an important factor for elucidating the mechanism of the onset of stroke and the functional mechanism of TJMs.

In this study, it was clear that onregedokuto, goreisain and shichimotsusukokato have the prolonging effect of the life span of SHRSP. Further study will be needed to verify whether the analysis of changing proteins can determine an association with the onset of stroke and its improving efficacy of TJMs.

Acknowledgements

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References