Effects of *Hange-shashin-to* on Cholera Toxin-Induced Fluid Secretion in the Small Intestine of Rats

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The effects of *Hange-shashin-to* (TJ-14) on cholera toxin-induced intestinal fluid secretion were studied to elucidate the mechanism by which this kampo medicine manifests anti-diarrheal effects. TJ-14 suppressed the intestinal fluid secretion induced by cholera toxin (1 μg/rat) in a dose-dependent manner at doses between 125 and 1000 mg/kg. It also inhibited the luminal prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) level. On the other hand, serotonin (5-HT) release was not affected by TJ-14. Subcutaneous injection of indomethacin at 10 mg/kg or ondansetron at 100 μg/kg significantly suppressed intestinal secretion. The luminal PGE<sub>2</sub> level was also inhibited by indomethacin (10 mg/kg, s.c.). TJ-14, even at 10<sup>-4</sup> g/ml, had little effect on the phasic contraction of isolated guinea pig ileum induced by 5-HT (2×10<sup>-6</sup> g/ml), while ondansetron suppressed the phasic contraction caused by 5-HT.

These results indicate that TJ-14 is useful in suppressing cholera toxin-stimulated intestinal fluid secretion, and that this effect is partially due to its suppressive action on the PGE<sub>2</sub> level.

Key words *Hange-shashin-to*; diarrhea; cholera toxin; prostaglandin E<sub>2</sub>; serotonin

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*Hange-shashin-to* (TJ-14) is often used to treat acute and chronic gastrointestinal catarrh, fermentative diarrhea and acute gastroenteritis. We have previously reported that TJ-14 is effective in treating diarrhea caused by castor oil, and that it does not markedly affect intestinal motility. Furthermore, TJ-14 suppressed the colorectal prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) level and enhanced colorectal water absorption. It is known that cholera toxin-induced diarrhea is a secretory diarrhea which involves serotonin (5-HT) and PGE<sub>2</sub>. Several studies have demonstrated the effectiveness of 5-HT receptor antagonists and indomethacin in suppressing this type of diarrhea. The present study was undertaken to elucidate the mechanisms by which TJ-14 manifests anti-diarrheal effects. For this purpose, we examined the effects of TJ-14 on cholera toxin-stimulated intestinal fluid secretion.

MATERIALS AND METHODS

**Animals** Eight-week-old male Wistar rats (SLC, Japan) and eight-week-old male Hartley guinea pigs (SLC, Japan) were used after a one-week acclimatization period. The animals were bred in quarters in which the temperature and relative humidity were kept at 23±2°C and 55±10% respectively, and which were lit between 7:00 and 19:00. The animals were allowed free access to water and drinking water.

**Test Drugs** TJ-14, a dried powder extract prepared by Tsumura Co., Ltd., and manufactured from a mixture of *Pinelliae Tuber* (relative quantity=5.0), *Scutellariae Radix* (2.5), *Glycyr rhiza Radix* (2.5), *Zizyph Fructus* (2.5), *Ginseng Radix* (2.5), *Zingiberis Siccatum Rhizoma* (2.5) and *Coptidis Rhizoma* (1.0), was dissolved in distilled water before use. The yield of the extract was 24% for TJ-14. Indomethacin, 5-HT and cholera toxin were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Ondansetron (Zofran Injection, Glaxo, U.K.) was also used. PGE<sub>2</sub> was quantified using a radio immunoassay (RIA) kit (NEN Co., Boston, U.S.A.). All other chemicals were of the highest grade commercially available.

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Effects on Cholera Toxin-Induced Intestinal Secretion

Ten to eleven animals were allocated into each group. The experiment was carried out according to the method of Beubler et al. Rats were deprived of food but allowed free access to water for 24 h. After celiotomy under urethane anesthesia (1.25 g/kg, i.p.), a polyethylene catheter (PE 60) was placed in the jejunum, about 5 cm distal from the flexura duodenojejunalis, and fixed by ligation. The jejunum was rinsed by infusing 2 ml of saline at 37°C into the catheter, then emptied via an incision about 25 cm distal to the catheter. The jejunal loop was then made by tying up the small intestine about 20 cm distal from the catheter. Either 2 ml of Tyrode’s solution or Tyrode’s solution plus cholera toxin (0.5 μg/ml) was injected into the jejunal loop. The animals were sacrificed 4 h later and the luminal fluid was measured. In the case of a lack of sufficient luminal fluid, 2 ml of Tyrode’s solution was injected again 30 min before the sacrifice. The net fluid transport was expressed as ml/h/g wet weight of jejunum. The net absorption was indicated by a negative value and net secretion by a positive value.

5-HT release was determined as follows. 0.9 ml of each sample was combined with 0.1 ml of 2 M HClO<sub>4</sub> containing isoprotenerol at a concentration of 1 μg/ml. The mixture was left on ice for more than 30 min, then centrifuged for 15 min at 20000 g. The supernatant (0.8 ml) was combined with 0.08 ml of 2 M CH<sub>3</sub>COONa. The mixture was then filtrated through a Millex-GV filter (0.45 μm). 5-HT was measured by HPLC-electron capture detector (ECD) using an Eicomp ECD 200 device, an Eicom CD-50DS column (4.6×150 mm), and an 80% 0.1 M phosphate–20% methanol buffer (pH 6.0). According to the method of Kobayashi et al., PGE<sub>2</sub> in the secreted fluid was quantified by the RIA kit. Electrolyte levels were measured using an automatic electrolyte analyzer (Model 710, Hitachi, Japan). TJ-14 (125—1000 mg/kg) was orally administered to the animals 1 h before the cholera toxin. Indomethacin was subcutaneously administered at 10 mg/kg 1 h before the cholera toxin. Ondansetron at 10 μg/kg was subcutaneously injected 15 min before and 2 h after the
cholera toxin treatment.

**Effects on 5-HT-Induced Contraction of Isolated Intestines**  This experiment was carried out according to a method described in detail elsewhere.**1** Briefly, Guinea pigs were sacrificed by exsanguination and the ileum part approximately 10 cm rostral to the ileocecum was resected. The ileum sample, cut into pieces about 2 cm in length, was used as the preparation. Each sample was suspended by a 1 g load (resting tension) in a 20 ml Magnus' tube filled with Tyrode's solution and was ventilated with a mixture of O₂ (95%) and CO₂ (5%). The composition of Tyrode solution was as follows (mm): NaCl, 137.9; KCl, 2.7; MgCl₂, 0.5; NaH₂PO₄, 1.1; CaCl₂, 1.8; NaHCO₃, 11.9 and glucose, 5.6. Experiments were carried out at 32 °C. The responses of the ileum samples were isotonically recorded via an isotonic transducer (ME-4012, MEC) using a pen-recorder (Uicorder, U-228, Nippon Denki Kagaku Co., Ltd.). Contraction responses were elicited by 5-HT (2×10⁻⁶ g/ml). Various concentrations of test drugs were applied to the preparation 5 min before the administration of 5-HT. The results are expressed as a percentage of the phasic contractions before drug treatment. Four to eight tests were carried out for each concentration of the drugs.

**Statistical Analysis**  All values are expressed as means ± S.E. The data from the in vivo study were evaluated by one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference procedure.

**RESULTS**

**I. Effects on Cholera Toxin-Induced Fluid Secrecion**

1) Effects on Fluid Transfer: Cholera toxin (1 μg/rat) markedly enhanced intestinal fluid secretion. This effect of the cholera toxin was suppressed by oral treatment with TJ-14 (125—1000 mg/kg) in a dose-dependent manner. Cholera toxin-stimulated intestinal fluid secretion was significantly suppressed by treatment with indomethacin (10 mg/kg) or ondansetron (100 μg/kg), as shown in Fig. 1.

2) Effects on Luminal PGE₂ Level: TJ-14 at doses of 125 to 1000 mg/kg suppressed the luminal PGE₂ level in a dose-dependent manner. Indomethacin (10 mg/kg) also markedly suppressed the luminal PGE₂ level, while ondansetron (100 μg/kg) did not suppress this parameter (Fig. 2).

3) Effects on Luminal 5-HT Level: TJ-14 did not markedly affect the 5-HT level, even at a dose of 1000 mg/kg. Indomethacin (10 mg/kg) and ondansetron (100 μg/kg) showed no marked effects on the 5-HT level (Fig. 3).

4) Effects on Electrolyte Levels in Luminal Fluid: In normal rats, the mean values of sodium, potassium and chloride showed net absorption in this experiment. Treatment with the cholera toxin reversed this net electrolyte absorption into net secretion. TJ-14 dose-dependently suppressed these changes in ion levels. Indomethacin (10 mg/kg) and ondansetron (100 μg/kg) also improved these changes to a similar degree, as shown in Table 1.

**II. Effects on Isolated Intestine**  Typical responses of TJ-14 (10⁻⁴ g/ml) and ondansetron (10⁻⁵ g/ml) on the phasic contraction induced by 5-HT (2×10⁻⁶ g/ml) in isolated guinea pig ileum are shown in Fig. 4. TJ-14 did not affect the 5-HT-induced contraction of the intestine, even at a dose of 10⁻⁴ g/ml, while ondansetron suppressed the intestinal contraction induced by 5-HT (Fig. 5).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>Net Na transfer μeq/h/g</th>
<th>Net K transfer μeq/h/g</th>
<th>Net Cl transfer μeq/h/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>10</td>
<td>-84.1 ± 8.1***</td>
<td>-0.3 ± 0.3***</td>
<td>-80.1 ± 7.8***</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>11</td>
<td>81.5 ± 6.3</td>
<td>5.2 ± 0.5</td>
<td>53.3 ± 5.0</td>
</tr>
<tr>
<td>TJ-14</td>
<td>125</td>
<td>10</td>
<td>73.0 ± 9.7</td>
<td>4.2 ± 0.8</td>
<td>53.0 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>10</td>
<td>69.7 ± 7.2</td>
<td>4.0 ± 0.4</td>
<td>44.7 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>10</td>
<td>32.9 ± 8.1***</td>
<td>2.6 ± 0.4***</td>
<td>22.3 ± 7.4***</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>10</td>
<td>27.7 ± 9.4***</td>
<td>2.8 ± 0.5***</td>
<td>13.4 ± 8.6***</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>11</td>
<td>44.3 ± 7.2**</td>
<td>3.1 ± 0.4***</td>
<td>22.5 ± 6.6***</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>0.1</td>
<td>11</td>
<td>32.7 ± 13.0***</td>
<td>2.8 ± 0.6**</td>
<td>10.7 ± 9.4***</td>
</tr>
</tbody>
</table>

Net electrolyte transfer was expressed in μeq/h/g wet weight. Results were expressed as means±S.E. Negative values denote absorption and positive values denote secretion. ** and ***: significantly different from the control at p<0.01 and p<0.001, respectively.

A : TJ-14 10⁻⁴ g/ml

B : Ondansetron 10⁻⁴ g/ml

Fig. 4. Typical Responses of TJ-14 (10⁻⁴ g/ml) and Ondansetron (10⁻⁵ g/ml) Regarding the Phasic Contraction Induced by 5-HT (2×10⁻⁶ g/ml) in Isolated Guinea Pig Ileum

Test drugs were applied to the preparation 5 min before the administration of 5-HT. Drug efficacies were evaluated as a percentage of the phasic contraction before drug treatment.

Fig. 5. Effects of TJ-14 and Ondansetron on the Phasic Contraction Induced by 5-HT (2×10⁻⁶ g/ml) in Isolated Guinea Pig Ileum

Ordinate scale expressed as a percentage of the phasic contraction before drug treatment. Each point represents the mean±S.E. of 4 to 8 experiments. ■, TJ-14; ○, ondansetron.

adequate digestion and absorption. A variety of factors can cause diarrhea. These include stress, bacterial toxins, cold stimulation, ingestion of food or drugs, and gastrointestinal diseases such as colorectal cancer and Crohn’s disease.⁹⁻¹¹ The diarrhea induced by cholerin toxin is a secretory type of diarrhea, which is known to cause a severe loss of bodily fluid and energy. The present study was conducted to assess the effects of TJ-14 on cholerin-toxin-induced fluid secretion.

It is known that cholerin-toxin-stimulated intestinal fluid secretion is related to an increase in cAMP. The increased cAMP inhibits the absorption of sodium and chloride, and thus leads to diarrhea.⁴,¹² 5-HT and PGE₂ also play an important role in cholerin-toxin-induced intestinal fluid secretion.⁴,¹² Some investigators have proposed mechanisms which explain the involvement of cAMP, 5-HT and PGE₂ in the pathophysiology of cholerin-stimulated fluid secretion.⁴,¹² Cholerin toxin may activate the adenylate cyclase-cAMP system in the enterochromaffin cells, resulting in 5-HT release. The released 5-HT then activates 5-HT₂ receptors which stimulate intestinal PGE₂ formation. 5-HT also directly activates the secretory nerve reflex arch through 5-HT₂ receptors.⁴,¹³,¹⁴ 5-HT-induced secretion has not been related to cAMP but has been thought to increase calcium gating in the epithelial cells and to activate intestinal nervous reflexes.¹⁵,¹⁶ PGE₂ enhances intestinal motility and inhibits Na⁺, K⁺-ATPase activity, thus suppressing the absorption of sodium and chloride, enhancing potassium secretion and reducing fluid absorption, leading to the onset of diarrhea.¹⁷—⁹) Regarding the characteristics of TJ-14’s antidiarrheal effects, we have previously reported that TJ-14 suppresses cas-

DISCUSSION

Diarrhea occurs when intestinal fluid secretion and peristalsis are enhanced and ingested foods are eliminated before
tor oil-induced diarrhea, despite its lack of effect on intestinal motility, and that it decreases the colorectal PGE$_2$ level in rats in a dose-dependent manner. Diarrhea induced by castor oil involves prostaglandins and platelet activating factor. In the present study, TJ-14 markedly suppressed the cholera toxin-stimulated PGE$_2$ level. These findings from the present and previous studies suggest that the inhibition of PGE$_2$ is involved in the antiarrheal effects of TJ-14. Further studies are needed to identify the components of TJ-14 which contribute to this effect. In this respect, it has been reported that *Scutellariae Radix*, *Glycyrrhiza Radix* and some components of *Zingiberis Scatuum Rhizoma* suppressed cyclooxygenase. Furthermore, berberine, a component of *Coptidis Rhizoma*, suppressed cholera toxin-stimulated intestinal fluid secretion. These previous findings seem to be related to the results obtained in the present study.

Several subtypes of the 5-HT receptor have been identified. The stimulation of intestinal fluid secretion by cholera toxin seems to involve both 5-HT$_3$ and 5-HT$_3$ receptors. In fact, both ketanserin and ICS205-930 (a 5-HT$_3$ receptor antagonist) are effective against cholera toxin-induced secretion in rats. Beubler et al. reported that the 5-HT$_3$ receptor-activated secretory response to cholera toxin depended on the release of prostaglandins as final mediators. 5-HT$_3$ receptors are located on neuronal structures. 5-HT$_3$ was also reported to induce a biphasic concentration–response curve in the guinea pig ileum. 5-HT$_3$ receptors mediate the first phase of the concentration–response curve, while 5-HT$_3$ receptors mediate the second phase. In the present study, the concentration of 5-HT applied to guinea pig ileum was thought to be mediated by 5-HT$_3$ receptors. TJ-14 did not suppress the 5-HT-induced contraction of the isolated intestines. On the other hand, ondansetron (a 5-HT$_3$ antagonist) suppressed cholera toxin-induced fluid secretion in 5-HT$_3$-induced intestinal contraction. Therefore, the effect of TJ-14 in suppressing cholera toxin-stimulated intestinal fluid secretion does not involve the blockade of 5-HT$_3$ receptors.

It is well known that cAMP is an important factor in cholera toxin-induced intestinal secretion. Ohmoto et al. reported that *Hange-shashin-to* showed inhibitory activity against cAMP phosphodiesterase. This inhibition would lead to an increase in intracellular cAMP levels. As mentioned above, cholera toxin may activate the adenylate cyclase in the enterochromaffin cells, which causes 5-HT release. Beubler et al. reported that endogenous PGE$_2$ is not involved in the cholera toxin-induced formation of cAMP, and that physiological doses of PGE$_2$ may act by facilitating calcium entry, rather than by increasing intracellular calcium through the activation of adenylate cyclase. Furthermore, some reports have indicated that indomethacin does not affect cholera toxin-induced cAMP formation. Although further investigations are necessary, it seems likely that the effects of TJ-14 do not depend on the suppression of cAMP in cholera toxin-stimulated intestinal secretion.

Diarrhea can be viewed as a defensive response designed to eliminate harmful substances from the body. Therefore, the condition of patients with cholera can be exacerbated if antiarrheal agents based on the suppression of intestinal motility are used. Drugs such as TJ-14, which suppress PGE$_2$ and enhance water absorption without affecting intestinal motility, may be useful in preventing the reduction of water absorption.

In summary, the present study revealed that the suppression of PGE$_2$ is involved in the antiarrheal effects of TJ-14. It was also suggested that TJ-14 can prevent the loss of water associated with diarrhea.

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**REFERENCES**