Effects of *Rikkunshi-to*, a Traditional Japanese Medicine, on the Delay of Gastric Emptying Induced by $N^G$-Nitro-L-arginine

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Abstract. We evaluated the effects of *Rikkunshi-to* and several of its ingredients on the delay of gastric emptying induced by a nitric oxide (NO) synthase inhibitor, $N^G$-nitro-L-arginine (L-NNA). After oral administration of L-NNA to rats, the gastric emptying rate at 24 h was decreased from $82.8 \pm 2.4\%$ to $53.3 \pm 5.7\%$. The decrease of the gastric emptying rate induced by L-NNA treatment was markedly ameliorated by administration of *Rikkunshi-to* (250 and 500 mg/kg, p.o.) in a dose-dependent manner. To identify the active ingredient of *Rikkunshi-to*, the components were separated according to polarity, and the effects of the respective fractions on gastric emptying were evaluated. Significant efficacy was found in the water and methanol fractions, but not in the 50% aqueous–methanol fraction. Furthermore, hesperidin (1–4.29 mg/kg, p.o.) contained in the methanol fraction and L-arginine (4.5 mg/kg, p.o.) contained in the water fraction ameliorated the decrease in the gastric emptying rate induced by L-NNA treatment. These results suggest that *Rikkunshi-to* ameliorated abnormalities of NO-mediated gastric functions such as delayed gastric emptying, and hesperidin and L-arginine were identified as two of the active ingredients contributing to the ability of *Rikkunshi-to* to facilitate gastric emptying.

Keywords: *Rikkunshi-to*, $N^G$-nitro-L-arginine, gastric emptying, hesperidin, L-arginine

Introduction

*Rikkunshi-to* (Chinese name Liu-Jun-Zi-Tang), one of the traditional Japanese medicines, is widely prescribed for patients with chronic hypofunction of the gastrointestinal tract, including gastric flatulence, anorexia, nausea, and vomiting. These gastrointestinal symptoms are closely associated with delayed gastric emptying in patients with chronic dyspepsia (1, 2). Tatsuta et al. reported that oral administration of *Rikkunshi-to* to patients significantly reduced chronic dyspepsia after 7 days of treatment and produced significantly better gastric emptying than did administration of a placebo (1). However, the mechanisms involved in gastric emptying are not well understood. Therefore, to clarify the role of *Rikkunshi-to* in gastric symptoms, we investigated the effect of *Rikkunshi-to* on gastric emptying using an experimental model.

Nitric oxide (NO) plays an important role in gastrointestinal systems (3–7). NO synthase (NOS) is present in the gastrointestinal nervous system, and NO has been proposed as a non-adrenergic non-cholinergic (NANC) mediator (3). Inhibition of NO production inhibits proximal stimuli-induced pyloric relaxation and enhances distal stimuli-induced pyloric excitation in anesthetized dogs (4). In addition, intravenous administration of $N^G$-nitro-L-arginine (L-NNA) prolongs the gastric emptying time in conscious dogs (5), and our previous study reported that the oral administration of L-NNA induced a delay in gastric emptying in rats (6, 7). These findings...
suggest that NO plays an important role in the gastrointestinal system and that the delay of gastric emptying is induced by decreased NOS activity.

In the present study, to elucidate the validity of Rikkunshito as a treatment for the symptom of chronic dyspepsia, we investigated the effect of Rikkunshito on delayed gastric emptying using an l-NNA-induced experimental model, and we also focused on identifying the active ingredients of Rikkunshito.

Materials and Methods

Drugs and chemicals

Rikkunshito was obtained from Tsumura & Co. (Tokyo) in the form of a dried powder extract. Rikkunshito was extracted from a mixture of Glycyrrhizae rhizoma (4.7%), Zingiberis rhizoma (2.3%), Atractylodis lanceae rhizoma (18.6%), Zizyphi fructus (9.3%), Aurantii nobilis pericarpium (9.3%), Ginseng radix (18.6%), Pinelliae tuber (18.6%), and Hoelen (18.6%). A voucher specimen (No. 280043010) has been deposited at our institute. l-NNA was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All ingredients contained in the methanol fraction of Rikkunshito were isolated in our laboratory.

Preparation of water, 50% aqueous-methanol, and methanol fractions from Rikkunshito

A spray-dried powder extract of Rikkunshito was dissolved in water. The extract was chromatographed on a highly porous polymer (Diaion HP20; Mitsubishi Chemical Co., Tokyo) and successively eluted with water, 50% aqueous-methanol, and methanol. The respective eluates were lyophilized. The yields of the water, 50% methanol, and methanol fractions were 70.8%, 9.8%, and 2.4%, respectively. In addition, the freeze-dried water fraction was dissolved in water, and then ethanol was gradually added to a final concentration of 80%. After centrifugation at 1400 × g for 20 min, the supernatant (80% ethanol-soluble fraction) and the precipitate (80% ethanol-insoluble fraction) were lyophilized. The yields of the 80% ethanol-soluble and -insoluble fractions were 56.1% and 43.9%, respectively.

Animals

Male SD rats, 6-week-old, were obtained from Charles River Japan, Inc. (Tokyo). The animals were used in the present study after acclimatization for 1 week. The animals were allowed free access to water and standard laboratory food and kept in a facility at a temperature of 24 ± 1°C, relative humidity of 55 ± 5%, and controlled lighting with lights on from 7:00 to 19:00 h daily. All animal experiments were performed in accordance with our institutional guidelines after obtaining the permission of the Laboratory Animal Committee. The experiments in the present study were designed to minimize the number of animals used and their suffering.

Analysis of ingredients in methanol fraction by high performance liquid chromatography

The ingredients in the methanol fraction were determined by high performance liquid chromatography (HPLC) with a photodiode-array detector (SPD-M10AVP; Shimadzu Co., Tokyo). The separation column was a TSK GEL ODS-80TS column (250 × 4.6 mm; Tosoh, Tokyo), and the column temperature was maintained at 40°C. Binary gradient elution was performed with mobile phase A (0.05 mol/L ammonium acetate-acetic acid buffer, pH 3.6) and mobile phase B (100% acetonitrile). The separation was initiated with 90% A and 10% B, and the concentration of B was linearly increased to 100% over a period of 60 min. The flow rate was set at 1.0 mL/min. The effluents from the column were detected at absorbances ranging from 200 to 420 nm using a photodiode-array detector. Data collection and processing for peak analyses were accomplished with CLASS-LC10 System Analysis Software (Shimadzu Co.).

Evaluation of gastric emptying

The experiment was conducted according to the method reported by Takeda et al. (8). To delay gastric emptying, 10 mg/kg of l-NNA was orally administered to rats 24 h before the evaluation of gastric emptying. All test drugs and fractions were dissolved in distilled water containing 1% Tween 80 and orally administered 6 and 22 h after l-NNA treatment. To evaluate the gastric emptying, 1 mL of phenol red (100 µg/mL) as a non-absorbable marker was injected into the stomach of the rat 24 h after l-NNA treatment. Each rat was sacrificed 15 min after phenol red administration, except for those sacrificed immediately to recover the whole dose of phenol red, and the stomach was removed immediately. The stomach was cut into several pieces in 10 mL of Na₂HPO₄ solution (0.1 mol/L) to collect the gastric contents and phenol red, and then 1 mL of the rinse solution was added to 2 mL of Na₂HPO₄ solution (0.1 mol/L) (S1). The residual rinse solution was added to 1 mL of phenol red (100 µg/mL) solution, mixed, and then diluted tenfold with Na₂HPO₄ solution (0.1 mol/L) (S2). The absorbances of S1 and S2 were measured as OD1 and OD2 at a wavelength of 560 nm.
UV-1200 spectrophotometer (Shimadzu Co.). The gastric emptying was calculated as follows:

Gastric emptying (%) = 100 - (A/B) × 100

A: The amount of phenol red remaining in the stomach (µg) = 100 - (3 × (OD1) × a + b) / (10 × (OD2) × a + b) / 3 × (OD1) × a + b - 1)

Note: a and b are coefficients obtained from the standard curve of phenol red.

B: The amount of phenol red recovered from the stomach immediately after phenol red administration (µg).

Statistical procedures

All data were expressed as the mean ± S.E.M. Statistical significance was measured by a one-way analysis of variance (ANOVA) followed by Fisher’s Protected Least Significant Difference test. Significance was accepted at P < 0.05.

Results

Delay of gastric emptying induced by L-NNA

Figure 1 shows the changes in gastric emptying induced by L-NNA orally administered 24 h before the evaluation of gastric emptying. The control group was orally administered distilled water at the same time. The gastric emptying rate observed in the treated group was markedly decreased from 78 ± 4.0% to 58.6 ± 4.7% by treatment with 10 mg/kg of L-NNA.

Effect of Rikkunshi-to on the delay of gastric emptying induced by L-NNA treatment

Figure 2 shows the effects of Rikkunshi-to administered orally 6 and 22 h after L-NNA (10 mg/kg) treatment on the decrease in the gastric emptying rate. The normal rate of gastric emptying (82.8 ± 2.4%) was significantly decreased to 53.3 ± 5.7% by L-NNA treatment, but the decrease was significantly ameliorated by administration of Rikkunshi-to in a dose-dependent manner (70.7 ± 3.3% and 81.7 ± 3.3% in 250 and 500 mg/kg treated rats, respectively). Metoclopramide (3 mg/kg) as a positive control also showed significant efficacy in this model.

Effects of fractional extracts of Rikkunshi-to on delayed gastric emptying

The effects of three fractions of Rikkunshi-to on the delayed gastric emptying induced by L-NNA are shown in Table 1A. Rikkunshi-to was fractionated according to lipophilicity using Diaion HP20 column chromatography. The effect of each fraction on the delayed gastric emptying was examined individually, at a dose equivalent to 500 mg/kg Rikkunshi-to. As shown in Table 1A, a marked effect on the decrease of the gastric emptying rate was found with the water (354 mg/kg) and methanol (12 mg/kg) fractions but not the 50% aqueous–methanol fraction (49 mg/kg). Furthermore, to clarify the active ingredient, the water fraction was divided into an 80% ethanol-soluble fraction (supernatant) and an insoluble fraction (precipitate), and the effects of these fractions were evaluated (Table 1B). Oral administration of the 80% ethanol-soluble fraction (198.5 mg/kg) and L-arginine (4.5 mg/kg) significantly ameliorated the decrease of the gastric emptying rate.

Effect of ingredients isolated from methanol fraction on delayed gastric emptying

A three-dimensional HPLC profile of the methanol fraction is shown in Fig. 3. The methanol fraction contained liquiritin apioside, liquiritin, liquiritigenin, isoliquiritin apioside, isoliquiritin, isoliquiritigenin, glycyrrhizin, narirutin, and hesperidin. Liquiritigenin is
an aglycon of liquiritin apioside and liquiritin, while isoliquiritigenin is an aglycon of isoliquiritin apioside and isoliquiritin. To compare the efficacy of these ingredients in Rikkunshi-to, the effects of 1 mg/kg liquiritigenin, isoliquiritigenin, narirutin, hesperidin, and glycyrrhizin on gastric emptying were measured and are shown in Table 2. Among these ingredients, only hesperidin ameliorated the delay of gastric emptying.

### Effect of hesperidin on the delay of gastric emptying
Hesperidin (0.1 – 4.29 mg/kg) dose-dependently improved the gastric emptying rate decreased by L-NNA treatment (Fig. 4). A significant increase in the emptying rate was observed at 1.0 and 4.29 mg/kg.

### Discussion
Rikkunshi-to, a gastroprotective herbal medicine, is effective for the symptoms of patients with chronic dyspepsia (9). Previously, the acceleration of gastric emptying (1, 10), the increase in plasma somatostatin and gastrin levels (11), and the stimulation of gastric myoelectric activity after gastrointestinal surgery (12) were reported as clinical effects of Rikkunshi-to. On the other hand, some basic studies have demonstrated that Rikkunshi-to prevents gastric mucosal damage (13, 14) and recovers the gastric adaptive relaxation (GAR) abolished by treatment with L-NNA in the isolated guinea pig stomach (15). These studies were designed to support clinical use, but basic studies regarding the effect of Rikkunshi-to on gastric emptying have not been reported. We focused on the effect of Rikkunshi-to on the gastric emptying using an experimental model. We found that administration of Rikkunshi-to significantly ameliorated in a dose-dependent manner the abnormality of gastric emptying induced by L-NNA treatment.

It has been reported that NO mediates relaxation of the gastric wall muscle or sphincter muscle (16 – 18) and plays an important role in gastrointestinal mucosal blood flow (19), protective (20) and reservoir functions such as GAR (15). Furthermore, Mashimo et al. reported that...
gastric emptying is significantly delayed in neuronal NOS (nNOS) knockout mice, but not in endothelial NOS (eNOS) knockout mice (21). These reports suggest that NO plays a role in regulating gastrointestinal motility such as the discharge and reservoir functions. On the other hand, it is reported that Rikkunshi-to recovered reservoir functions such as GAR that were abolished by treatment with L-NNA (15) and that the gastroprotective effect of Rikkunshi-to against ethanol-induced mucosal injury was inhibited by pretreatment with L-NNA (13, 14). It has been suggested that impairment of the proximal gastric relaxation response causes antral distention, resulting in disturbance of gastric emptying (22). GAR may be one of the mechanisms of gastroprotection (23). Therefore, these reports and our findings suggest that the mechanism of Rikkunshi-to may be related to the NO pathway.

Rikkunshi-to consists of eight constituent medical herbs (described in Materials and Methods) and contains many components. We previously reported that the administration of Zingiberis rhizome (6) and Atractylodis lanceae (7) extract at dose of 250 mg/kg shows remarkable efficacy in this model. To identify the active ingredient of Rikkunshi-to, we compared the activity of three fractions (water, 50% aqueous-methanol, and methanol fraction) of Rikkunshi-to and observed a significant efficacy in the water and methanol fractions, but not in the 50% aqueous-methanol fraction.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Gastric emptying (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>82.1 ± 3.6</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>54.8 ± 6.8**</td>
</tr>
<tr>
<td>Liquiritigenin</td>
<td>1.0</td>
<td>59.5 ± 5.9*</td>
</tr>
<tr>
<td>Isoliquiritigenin</td>
<td>1.0</td>
<td>60.9 ± 7.4</td>
</tr>
<tr>
<td>Narirutin</td>
<td>1.0</td>
<td>52.9 ± 8.3</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>1.0</td>
<td>81.8 ± 2.3**</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>1.0</td>
<td>59.0 ± 6.7</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.E.M. of 8 or 9 animals. **Significantly different from the control group at P<0.01. *Significantly different from the normal group at P<0.01.
Therefore, the water fraction was separated according to solubility in 80% ethanol. The supernatant (80% ethanol-soluble fraction) contains low-molecular-weight components, organic acids, and amino acids such as L-arginine, and the precipitate (80% ethanol-insoluble fraction) is polysaccharide-rich. We found significant efficacy in the supernatant but not in the precipitate (Table 1B).

L-Arginine, which is contained in Rikkunshi-to at a concentration of 0.9% (4.5 mg/500 mg of Rikkunshi-to), also ameliorated the delay of gastric emptying at dose of 4.5 mg/kg. It is recognized that L-arginine restores the changes in various physiological functions induced by treatment with an NOS inhibitor such as L-NNA (24). The L-arginine contained in Rikkunshi-to is separated in the supernatant from the water fraction. Thus, L-arginine may partially contribute to the effect of Rikkunshi-to and the water fraction in this model using an NOS inhibitor.

On the other hand, the methanol fraction contains low polarity components such as liquiritigenin, glycyrrhizin, narirutin, and hesperidin (Fig. 3). In order to identify the ingredients that contribute to the effect of Rikkunshi-to, we compared the efficacy of these ingredients using the same dose (1 mg/kg). Only hesperidin showed clear efficacy (Table 2). The amounts of hesperidin, narirutin, glycyrrhizin, liquiritigenin, and isoliquiritigenin in 500 mg of the Rikkunshi-to used in this study were 4.29, 1.01, 2.56, 1.59, and 0.156 mg, respectively. We evaluated glycyrrhizin at doses of 2.56 and 5 mg/kg and liquiritigenin at doses of 1.59 and 3 mg/kg, but did not observe significant efficacy (data not shown). However, we do not think that the effect of Rikkunshi-to is due to the activities of L-arginine and hesperidin only. In fact, the 6-gingersulfonic acid (1 mg/kg) and shogasulfonic acid A (0.3 mg/kg) contained in Zingiberis rhizome and the atractyloidin (0.1 mg/kg) contained in Atractylodis accelerated gastric emptying in this model (6, 7). Although the concentrations of these ingredients in Rikkunshi-to were lower than the doses at which activity is shown, a synergistic effect may have occurred. Hence, examination of the combination effects is required.

Hesperidin is a flavone glycoside derived from Aurantii nobilis pericarpium, one of the constituent medical herbs of Rikkunshi-to, and is found in many citrus fruits such as oranges. Hesperidin scavenges free radicals (25) and has anti-inflammatory (26) and anti-tumor (27) effects. Moreover, it is reported that hesperidin suppresses production of nitric oxide and expression of inducible NOS (iNOS) in an LPS-stimulated mouse macrophage cell line (28). Sakata et al. suggested that these inhibitory effects of hesperidin might be related to its anti-inflammatory and anti-carcinogenic effect (28). Thus, the improvement of gastric emptying due to hesperidin is not due to an increase in the supply of NO. In addition, it is widely known that NO is generated from the terminal guanidine nitrogen of L-arginine through the action of NOS. Hesperidin does not have a guanidine group (26). Therefore, we hypothesize that hesperidin is not a substrate for NO production by NOS and has a different mechanism from L-arginine. Moreover, metoclopramide (a dopamine D2-receptor antagonist, 5-hydroxytryptamine (5-HT3) antagonist, and 5-HT4...
agonist) also accelerated gastric emptying in this model. Thus, the mechanism of hesperidin also may be related to serotonergic or dopaminergic activities. In conclusion, Rikkunshi-to may ameliorate the symptom of chronic dyspepsia caused by an interruption of an NO-mediated system by recovering the discharge function, and hesperidin and L-arginine were identified as active ingredients of Rikkunshi-to.

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References


