Effects of Ninjin-to on Levels of Calcitonin Gene-Related Peptide and Substance P in Human Plasma

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The herbal medicine Ninjin-to has been used for the treatment of gastroenteritis, esogastritis, gastric atony, gastrectasis, vomiting, and anorexia. One of the mechanisms of the empirical effects is assumed to be due to local changes in neuropeptide levels. Sensory afferent neurons in the gastrointestinal mucosa regulate neuropeptides [calcitonin gene-related peptide (CGRP), substance P, etc.], which play various physiologic roles. To determine whether the pharmacologic effects of Ninjin-to on the gastrointestinal mucosa are due to changes in gastrointestinal mucosa regulatory peptide levels, we examined the effects of Ninjin-to on the levels of CGRP-like immunoreactive substances (IS) and substance P-IS in plasma taken from five healthy subjects. A single oral administration of 6.0 g of Ninjin-to caused significant increases in plasma CGRP-IS at 40 min and 60 min, and in substance P-IS levels at 90 min, compared with a placebo group. These results may indicate that the pharmacologic actions of Ninjin-to are closely related to changes in CGRP-IS and substance P-IS levels.

Key words Ninjin-to; calcitonin gene-related peptide; substance P; blood flow; peristaltic reflex

Postoperative ileus following laparotomy is a common complication after abdominal surgery and involves an inhibition of bowel transit caused by an impairment of motility. For postoperative ileus, some herbal medicines with experiential gastrointestinal effects have been elucidated pharmacologically. One of them, Dai-kenchu-to, is known to increase gastrointestinal motility and improve ileal function. It is thought that the part of the contractile mechanism is mediated by acetylcholine (ACh) release from the ends of cholinergic nerves. Furthermore, these effects are reported to cause significant increases in the levels of brain-gut peptides such as motilin and vasoactive intestinal peptide (VIP) in human plasma.

Ninjin-to is prepared from four crude drugs: Ginseng radix, Glycyrrhizae radix, Atractylodis rhizome, and Zingiberis siccatum rhizoma. This medicine has been used for the treatment of gastroenteritis, esogastritis, gastric atony, gastrectasis, vomiting, and anorexia. Ninjin-to is also reported to enhance gastrointestinal motility, similar to the gastrointestinal prokinetic drugs like cisapride and metoclopramide. Moreover, previous study has shown that Ninjin-to not only significantly improved gastrointestinal motility but also showed stronger effects than those of Dai-kenchu-to in a rat model of postoperative ileus. Hence, as well as Dai-kenchu-to, Ninjin-to may be an effective herbal medicine for postoperative ileus.

Materials and Methods

Materials Ninjin-to (EK-32, lot 01BJ), prepared as a 3.0-g dried powder extract in the proportion: Ginseng radix (3.0 g), Glycyrrhizae radix (3.0 g), Atractylodis rhizoma (3.0 g), Zingiberis siccatum rhizoma (3.0 g), was kindly supplied by Kanebo Co. Ltd. (Tokyo, Japan). A mixture of sucrose and cellulose was used as a placebo. Synthetic human CGRP and its fragment (8–37), substance P, were purchased from Biogenesis (Poole, U.K.). All other reagents were of analytical grade from commercial sources.

Subjects Five healthy male volunteers (nonsmokers) participated in this study. Each subject received information about the study’s scientific purpose, which was approved by the Ethics Committee of Oita Medical University, and gave informed consent. No subject had received any medication for 1 month preceding the test and no stimulator of gastrointestinal motility was administered to any subject during the study.

Study Schedule Ninjin-to or placebo was orally administered as a single dose of 6.0 g with water. Venous blood samples (10 ml) from a forearm vein were taken for EIA of the CGRP and substance P levels. Samples were taken 20, 40, 60, 90, 120, 180, and 240 min after administration of the
test substances. All subjects ate lunch at 11:30—12:00 and the study was carried out from 14:00 (2 h after lunch) to 18:00.

Preparation of Plasma Extracts The blood samples were collected in a chilled tube containing aprotenin (500 KIU/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). After centrifugation, the plasma samples were diluted with 4% acetic acid (pH 4.0) and loaded onto Sep-Pak C_{18} cartridges (Millipore Corp., Milford, U.S.A.), and washed with 4% acetic acid. The peptides in the plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0), lyophilized, and reconstituted to 100 μl with the assay buffer and subjected to EIA. For the EIA system, plasma samples were concentrated five-fold with Sep-Pak C_{18} cartridges. The recovery of plasma CGRP-IS and substance P-IS was >90% with this extraction procedure.

EIA of CGRP and Substance P ELAs for CGRP-14 and substance P-IS15 were performed using a delayed-addition method. Human CGRP (8—37) and substance P were conjugated with β-D-galactosidase (Boehringer Mannheim, Mannheim, Germany) with N-(ε-maleimidocaproyloxy)-succinimide.16 The assay was performed using a delayed-addition method, and separation of bound and free antigen was performed on an anti-rabbit IgG (55641) (ICN Pharmaceuticals, OH, U.S.A.-coated immunoplate. The fluorescence intensity of the fluorescent product 4-methylumbelliferone was measured on an anti-rabbit IgG (55641) (ICN Pharmaceuticals, OH, U.S.A.-coated immunoplate. The fluorescence intensity of the fluorescent product 4-methylumbelliferone was measured with an MTP-100 microplate reader (Corona Electric, Ibaraki, Japan). The enzyme immunoassays for CGRP and substance P were sensitive to detection limits of 0.08 and 0.40 fmol/well, respectively.

Data Analysis All values are expressed as the mean±S.D. Comparisons of plasma peptide levels among blood sampling times was made with analysis of variance and the Mann–Whitney U-test. Values of p<0.01 and p<0.05 were regarded statistically significant.

RESULTS AND DISCUSSION

Ninjin-to has been used for thousands of years to improve gastrointestinal functions. In recent years, it has been reported that Ninjin-to significantly improves gastrointestinal motility and it may be useful for the treatment of postoperative ileus.17 Although the underlying mechanisms on Ninjin-to remain unclear, studies by Naito et al. have shown that the effects of gastrointestinal motility are mainly regulated by hormonal and neuronal mechanisms, which may be related to brain-gut peptides (mainly motilin and somatostatin).18 Therefore, in this study, CGRP and substance P, which regulate gastrointestinal motility, were examined to study the effects of Ninjin-to.

The profiles of mean plasma CGRP-IS levels against time in five healthy subjects after a single oral administration of Ninjin-to or placebo are shown in Fig. 1. The data were compared with each mean value. Ninjin-to caused significant increases in CGRP-IS at 40 and 60 min (36.0±9.5 pg/ml at 40 min, 100.2±62.3 pg/ml at 60 min) compared with the response in the placebo group. Figure 2 shows plasma substance P-IS levels after the administration of Ninjin-to. Ninjin-to increased substance P-IS levels at 90 min (48.8±10.7 pg/ml) compared with the placebo. These results indicate that the action of Ninjin-to may be related to CGRP and substance P.

CGRP has several potent biological activities, including vasodilation, and in the gastric mucosa its vasodilatory effects following stimulated release from the extrinsic sensory innervation is considered to serve as an important protective mechanism for maintaining mucosal integrity.17—19 In addition, CGRP is also present in enteric neurons20 and has a potent effect on gastrointestinal motility and secretion.21 The localization of CGRP in the small intestine suggests a regulatory role for CGRP in small intestinal function. Previous studies have reported that the intrinsic sensory pathway, which mediates the peristaltic response to mucosal stimulation, utilizes CGRP as a sensory transmitter.22 Furthermore, CGRP is a potent intestinal vasodilator in conscious dogs and causes increases in the intestinal blood flow.23 It was reported that intraduodenal administration of Dai-kenchu-to increases intestinal blood flow in a dose-dependent manner that is mainly mediated by CGRP.24 Interestingly, Ninjin-to contains two of the same herbs as Dai-kenchu-to: Ginseng radix and Zingiberis siccatum rhizoma, both herbal medicines that have been used to improve gastrointestinal functions. In our results, Ninjin-to significantly raised plasma CGRP-IS levels 40 and 60 min after administration in healthy subjects. Accordingly, the mechanisms of Ninjin-to, as well as those of Dai-kenchu-to, based on the increment of CGRP-IS levels in human plasma, may include not only the improvement of peristaltic contractions but also increments in intestinal blood flow, both of which are mediated by CGRP.

Substance P coexists with CGRP in the sensory afferent neurons of the gastrointestinal mucosa and is released with ACh in response to depolarizing stimulation in the enteric nervous system.25 In the intestine, substance P controls...
motility and secretion. 25) Previous studies have shown that substance P neurons project into the myenteric plexus and circular muscle layer and may be involved in the regulation of the ascending contractile component of the peristaltic reflex. 26–28) Furthermore Schmidt et al. reported that administration of tachykinins/substance P to healthy volunteers increased intestinal mucosal blood flow. 29) Although whether substance P actually participates in the physiologic regulation of mucosal blood flow in the gastrointestinal tract is still not known, substance P may also be involved in regulation of the peristaltic reflex and of the mucosal blood flow in the intestine along with CGRP. In our results, Ninjin-to raised plasma substance P-IS levels after administration to healthy subjects. Ninjin-to may act in the gastrointestinal system and part of its action may be closely related to changes in substance P-IS levels in plasma.

In previous reports, Naito et al. assumed that Ninjin-to increases plasma somatostatin and motilin levels to improve gastrointestinal motor dysfunction and intracellular communication between somatostatin and motilin. 30) Somatostatin, a polypeptide widely distributed in the gastrointestinal tract, participates in the control of gut motility by exerting both inhibitory and stimulating influences. It is known that CGRP stimulates gastric somatostatin secretion and its actions are due to a direct effect on the gastric somatostatin-producing D-cells. 31) Somatostatin is also present in a small population of neurons that project within the myenteric plexus and appears to play a part in the sensory nervous system. Somatostatin neurons appear to act as facilitatory interneurons in intestinal peristalsis. 32) Motilin is a powerful inducer of motor activity in the stomach and intestine. It is reported that motilin-producing cells are innervated by local or intramural cholinergic neurons. 33) The previous reports and our results demonstrate that Ninjin-to stimulates CGRP, substance P, and somatostatin release from nerve endings and motilin release through muscarinic receptors on motilin cells in the enteric nervous system exert its pharmacologic activity.

The herbs contained in Ninjin-to may have an effect on neuropeptides. Ninjin-to has Zingiberis siccatum rhizoma as one of its ingredients, and this herb contains 6-gingerol and 6-shogaol as bioactive components, both of which have vanilloid structures. Capsaicin, the pungent ingredient in red peppers and a vanilloid receptor agonist, stimulates capsaicin-sensitive afferent neurons, which release CGRP and substance P from their nerve endings. 34–36) It has been reported that 6-shogaol produces an increase in intestinal blood flow, and that the CGRP receptor antagonist, CGRP (8–37), completely abolishes Ginger rhizome and 6-shogaol-induced hyperemia. 37) Naito et al. also reported that plasma CGRP and substance P levels significantly increased after administration of Zingiberis rhizoma extract to healthy subjects. Therefore these results suggest that 6-gingerol and 6-shogaol are the predominant ingredients to stimulate CGRP and substance P release from nerve endings.

We conclude that Ninjin-to may improve gastrointestinal motility and mucosal blood flow by significantly increasing CGRP and substance P-IS levels in plasma. Although further studies are needed to elucidate the mechanism, our findings indicate that Ninjin-to is closely related to changes in CGRP-IS and substance P-IS levels in plasma and it may be useful for the treatment of postoperative ileus.

REFERENCES