

Bushi-richu-to Raises Calcitonin Gene-related Peptide, Substance P, Somatostatin, and Vasoactive Intestinal Polypeptides Levels in Human Plasma

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The traditional Japanese *Kampo* medicine Bushi-richu-to (Bushi-ninjin-to) has been empirically used for the treatment of chronic gastroenteritis, gastric atony, and chronic constipation accompanied by a feeling of coldness in the body. One of the mechanisms of the empirical effects is assumed to be due to local changes in gut-regulated peptide levels. We studied the effect of Bushi-richu-to on calcitonin gene-related peptide (CGRP)-, substance P-, vasoactive intestinal polypeptides (VIP)-, somatostatin-, and motilin-like immunoreactive substances (IS) in plasma taken from healthy subjects. Bushi-richu-to (4.5 g) or placebo was orally administered to five healthy males. Blood samples were taken before, and at 20, 40, 60, 90, 120, 180, and 240 min after administration, extracted, and then submitted to a highly sensitive enzyme immunoassay system for CGRP-, substance P-, VIP-, somatostatin-, and motilin-IS. A single oral administration of Bushi-richu-to caused significant increases in plasma CGRP-, substance P-, VIP-, and somatostatin-IS levels compared with placebo. It is concluded that Bushi-richu-to might improve a peripheral and uncomfortable feeling of cold and gastrointestinal dysmotility via gut-regulated peptide release.

Key words — Bushi-richu-to, calcitonin gene-related peptide, substance P, vasoactive intestinal polypeptides, somatostatin, gastrointestinal motility

INTRODUCTION

Traditional herbal medicines have been employed for thousands of years and have contributed greatly to the treatment of many subjective symptoms. Bushi-richu-to (Bushi-ninjin-to) is a *Kampo* medicine (traditional Japanese medicine) consisting of five crude drugs; *Aconiti tuber*, *Ginseng radix*, *Glycyrrhizae radix*, *Atractylodis rhizome*, and *Zingiberis siccatum rhizome*. In *Kampo* medical care, this formula is traditionally used to treat chronic hypofunction of the gastrointestinal tract. It has been reported that Bushi-richu-to has clinical usefulness in the treatment of gastrointestinal motility disorders such as chronic constipation due to antipsychotic drugs and a peripheral and uncomfortable feeling of cold (*hie*).^{1,2)} There are some reports indicating that *Kampo* medicines containing both *Ginseng radix* and *Zingiberis siccatum rhizome* are effective herbal medicines for intestinal ischemia-related diseases or gastrointestinal motility disorders.^{3,4)} *Aconiti tuber* has been also used to improve the health of persons with a weak constitution and metabolism. *Aconiti tuber* has been described as having positive inotropic effects.⁵⁾

Gastrointestinal motility is mainly regulated by two factors, humoral hormones and nervous transmitters from both the central nervous system and peripheral enteric nervous system. Gut-regulated peptides exert actions on gastrointestinal motility via both endocrine hormones and peptidergic transmitters.⁶⁾ These peptides as modulatory mediators appear to be major components of bodily integration and have important regulatory actions on the physiological function of the gastrointestinal tract. Recent studies have indicated that some disorders of gastrointestinal motility following surgery⁷⁾ and chronic stresses⁸⁾ are related to gut-regulated peptides, such as motilin, vasoactive intestinal polypeptides (VIP), calcitonin gene-related peptide (CGRP), and substance P.

In recent years, some *Kampo* medicines have been elucidated pharmacologically from the viewpoint of gut-regulated peptide levels. Dai-kenchu-to, Ninjin-to and Keishi-ninjin-to affect gastrointestinal motility and based on empirical evidence their effects are assumed to be an alteration of gut-regulated peptides levels in healthy human plasma.^{9–11)} Among these drugs, it is reported that Ninjin-to potentially enhances gastrointestinal motility in a manner similar to that of gastrointestinal prokinetic drugs like cisapride and meto-

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clopramide.¹²⁾ Ninjin-to and Bushi-richu-to contain the same herbs (*Ginseng radix*, *Glycyrrhizae radix*, *Atractylodis rhizome*, and *Zingiberis siccatum rhizome*). Thus, we hypothesized that Bushi-richu-to could induce alterations of gut-regulated peptides in plasma, which could be involved in the pathogenesis of the gastrointestinal dysfunction. The purpose of this study was to determine whether the clinical effects of Bushi-richu-to on the gastrointestinal are due to changes in the plasma CGRP-, substance P-, VIP, somatostatin-, and motilin-like immunoreactive substance (IS) levels after the administration of Bushi-richu-to.

MATERIALS AND METHODS

Drugs and Chemicals— Spray-dried powdered Bushi-richu-to (EK-410) was purchased from Kanebo Co. Ltd. (Tokyo, Japan). The ratio of the respective herbs was as follows: *Aconiti Tuber*, *Ginseng Radix*, *Glycyrrhizae radix*, *Atractylodis rhizome*, and *Zingiberis siccatum rhizome*, in the ratio of 1 : 3 : 3 : 3 : 3 (4.5 g). The dosage of Bushi-richu-to was determined based on the maximum daily dose used in clinical therapy. The placebo was crystalline cellulose and lactose, in the ratio of 1 : 1. Synthetic human motilin, somatostatin, VIP, CGRP and its fragment (8–37), and substance P were purchased from the Peptide Institute (Osaka, Japan). The VIP fragment peptide was supplied by Professor H. Yajima (Kyoto University, Kyoto, Japan). Antisera to VIP (A604/R1B) and CGRP (CA1132) were purchased from Biogenesis (Poole, U.K.), somatostatin (T-4101) from Peninsula Laboratories (San Carlos, CA, U.S.A.), motilin (Y121) from Yanaihara Institute (Shizuoka, Japan), and substance P (RA-08-095) from Cambridge Research Biochemicals (Cambridge, U.K.). Goat affinity-purified antibody to rabbit IgG (whole molecule) (55641) was purchased from ICN Pharmaceuticals (Aurora, OH, U.S.A.). All other reagents were of analytical reagent grade from commercial sources.

Subjects— Five healthy male volunteers (non-smokers), aged 24–29 years (median 26 years), weighing 55–68 kg (median weight 62 kg), participated in this study. Each subject received information about the scientific purpose of the study, which was approved by the Ethics Committee of Oita Medical University, and gave their informed consent. No subject had received any medication for 1 month preceding the test and no stimulator

of gastrointestinal motility was administered to any subjects during the study.

Study Protocol— This was a two-way (Bushi-richu-to and placebo) crossover study. Bushi-richu-to (4.5 g) or placebo was orally administered with 100 ml water. Each subject was administered these drugs with an interval of four weeks. Venous blood samples (10 ml) were obtained from a forearm vein just before the drug was administered, and at 20, 40, 60, 90, 120, 180, and 240 min after administration of the test substances. The study was carried out from 14:00 to 18:00.

Preparation of Plasma Extracts— The blood samples were collected in a chilled tube containing aprotinin (500-kallikrein inhibitor units/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 C₁₈ cartridges (Millipore Corp., Milford, CA, 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 μ l with the assay buffer and subjected to enzyme immunoassay (EIA). For the EIA system, plasma samples were concentrated five-fold with Sep-Pak C₁₈ cartridges. The recovery of plasma CGRP-, substance P-, VIP-, motilin-, and somatostatin-IS was > 90% with this extraction procedure.

EIAs of CGRP, substance P, VIP, motilin, and somatostatin— EIAs for CGRP-,¹³⁾ substance P-,¹⁴⁾ VIP-,¹⁵⁾ motilin,¹⁶⁾ and somatostatin-IS¹⁷⁾ were performed as previously described, using a delayed-addition method.¹⁸⁾ Separation of bound and free antigen was performed on anti-rabbit IgG-coated immunoplates. The fluorescent product 4-methylumbelliferon was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan). Human somatostatin, porcine motilin, fragment VIP (11–28), human CGRP (8–37), and substance P were conjugated with β -D-galactosidase (Boehringer Mannheim, Mannheim, Germany) with *N*-(ϵ -maleimidocaproyloxy)-succinimide according to the method of Kitagawa *et al.*¹⁹⁾ The EIAs for somatostatin, motilin, VIP, CGRP, and substance P were specific and highly sensitive to detection limits of 0.10, 0.80, 1.00, 0.08 and 0.40 fmol/well, respectively.

Statistical Analysis— Data were analyzed using the SPSS 15.0J for Windows statistical software package (SPSS Japan Inc., Tokyo, Japan). All val-

ues are expressed as the mean \pm S.D. Statistical significance was evaluated using the Dunnett's-test. A value of $p < 0.01$ or $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS AND DISCUSSION

The plasma CGRP-IS level-time profile after administration of Bushi-richu-to to healthy subjects is shown in Fig. 1A. Bushi-richu-to significantly increased CGRP-IS at 40, 60, and 180 min (85.2 ± 58.7 pg/ml at 40 min, 62.3 ± 29.3 pg/ml at 60 min, and 85.2 ± 55.6 g/ml at 180 min, respectively) compared with the response of the placebo group (14.9 ± 1.9 pg/ml at 40 min, 17.0 ± 6.6 pg/ml at 60 min, and 25.0 ± 8.3 pg/ml at 180 min, respectively).

The plasma substance P-IS level-time profile after administration of Bushi-richu-to to healthy subjects is shown in Fig. 1B. Bushi-richu-to significantly increased substance P-IS at 180 min (68.5 ± 18.7 pg/ml) compared with the placebo group (34.3 ± 17.9 pg/ml), but later than plasma CGRP-IS levels. The plasma somatostatin-IS level-time profile after administration of Bushi-richu-to to healthy subjects is shown in Fig. 1C. Bushi-richu-to significantly increased somatostatin-IS at 60 and 90 min (20.2 ± 6.1 pg/ml at 60 min, and 19.1 ± 3.6 pg/ml at 90 min, respectively) compared with the placebo group (9.8 ± 2.1 pg/ml at 60 min, and 9.5 ± 2.3 pg/ml at 90 min, respectively). Bushi-richu-to did not, however, alter the levels of motilin (Fig. 1D). The plasma VIP-IS level-time profile after administration of Bushi-richu-to to healthy subjects is shown in Fig. 1E.

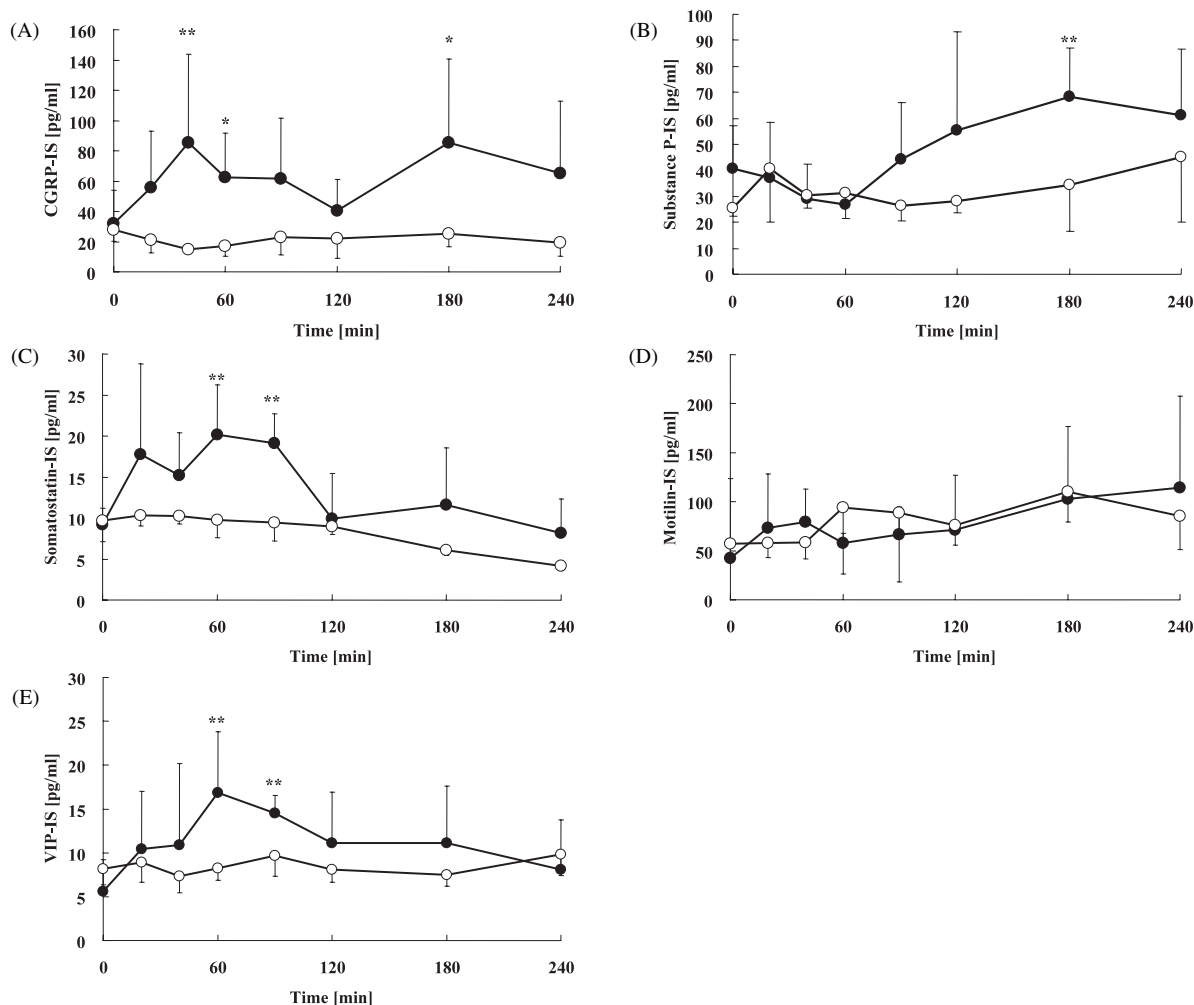


Fig. 1. Changes in Plasma CGRP-(A), Substance P-(B), Somatostatin-(C), Motilin-(D), and VIP-immunoreactive Substance (IS) Levels (E) After Oral Administration of Bushirichuto 4.5 g (●) or Placebo (○)
Each point represents the mean \pm S.D. of five subjects. ** $p < 0.01$ and * $p < 0.05$ compared with placebo.

Bushi-richu-to significantly increased VIP-IS at 60 and 90 min (16.9 ± 7.0 pg/ml at 60 min, and 3.7 ± 2.2 pg/ml at 90 min, respectively) compared with the placebo group (8.3 ± 1.4 pg/ml at 60 min, and 9.7 ± 2.3 pg/ml at 90 min, respectively).

CGRP has several potent biological activities, including vasodilation, and in the gastrointestinal 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.²⁰⁾ In addition, CGRP has a potent effect on gastrointestinal motility and secretion.²¹⁾ 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⁾ In this study, Bushi-richu-to significantly raised plasma CGRP-IS levels. Bushi-richu-to contains *Zingiberis siccatum rhizome* as one of its gradients. This herb contains 6-gingerol and 6-shogaol as bioactive components. It was reported that intraduodenal administration of 6-shogaol increases intestinal blood flow in a dose-dependent manner that is mainly mediated by CGRP.⁷⁾ It is known that this herb or some *Kampo* medicines which include this herb increased plasma CGRP-IS levels in human subjects.^{11, 24, 25)} Accordingly, Bushi-richu-to may also directly stimulate CGRP-containing nerves or indirectly secrete CGRP accompanied by the stimulation of other secretory cells and mechanisms. The mechanisms of Bushi-richu-to, based on the increment of CGRP-IS levels in human plasma, might 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.²⁶⁾ In the intestine, substance P controls motility and secretion. 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.²⁷⁾ In this study, Bushi-richu-to raised plasma substance P-IS levels. Bushi-richu-to might act in the gastrointestinal system and part of its action might be closely related to changes in substance P-IS levels in plasma. However, these se-

cretions of substance P showed delayed action in comparison with changes in CGRP. Although another pharmacologic mechanism might also affect neuropeptide levels due to a difference in the increasing time between CGRP and substance P, it is difficult to elucidate its reason from the results of the present study. Hence, we decided to determine the mechanism action of Bushi-richu-to in a small animal model using the rat.

VIP is widely distributed in the central and peripheral nervous system. This peptide has a vasodilating effect and increases peripheral blood flow. VIP is also known as a major regulator of mammalian intestinal motility and induces relaxation of precontracted ileal longitudinal muscle, and mediates its peristaltic reflex.^{28, 29)} In our results, Bushi-richu-to transiently increased plasma VIP-IS levels. This effect might be due to the *Aconiti Tubers* present in Bushi-richu-to. Preparations of *Aconiti Tuber* have been therapeutically used to increase peripheral body temperature and treat a peripheral and uncomfortable feeling of cold (*hie*). This herb contains aconite alkaloids (aconitine, mesaconitine, etc.) as its bioactive components. In a previous study, it was reported that mesaconitine elicited a strong relaxation in isolated rat aorta, and this relaxation is mainly endothelium-dependent and mediated by nitric oxide (NO), a powerful vasodilator agent.³⁰⁾ It is known that NO is a neuronal mediator of VIP/cholinergic vasodilation, and colocalized with VIP in the myenteric plexus.³¹⁾ Nagano *et al.* reported that Dai-kenchu-to significantly increased plasma VIP levels in healthy subjects and that all subjects reported some feeling warm.³²⁾ Although it is not known whether *Aconiti Tuber* directly stimulates VIP-containing nerves, this herb might be closely related to the release of VIP in the autonomic nervous system. The effects of Bushi-richu-to, based on the increment of VIP-IS levels in human plasma, may include improvements in "*hie*" and bowel obstruction.

Somatostatin, a polypeptide widely distributed in the gastrointestinal tract, participates in the control of gut motility by exerting both inhibitory and stimulating influences. In the interdigestive state somatostatin induces phase-3 activity and which in the digestive state it inhibits gastric emptying and slows gastrointestinal transit.³³⁾ Somatostatin also appears to act on facilitatory interneurons in intestinal peristalsis.³⁴⁾ In addition, somatostatin inhibits the secretion of gastrin, gastric acid, and motilin.^{35, 36)} In the present study, Bushi-richu-to significantly raised

plasma somatostatin-IS levels. Kawashima *et al.* reported CGRP increased somatostatin secretion and decreased gastric acid secretion via somatostatin-induced reduction of gastrin and histamine.³⁷⁾ In our study, plasma CGRP-IS levels were significantly increased earlier than plasma somatostatin-IS levels. It is thought that the stimulatory effect of Bushi-richu-to on CGRP-IS secretion might be due, at least in part, to an effect on somatostatin-IS levels. Increased somatostatin might correspond with gastrointestinal motor regulation including the acceleration of gastric emptying and peristaltic reflex, by this drug.

Motilin participates in regulating gastrointestinal motility, and stimulates gastric emptying and postprandial gastric contraction.³⁸⁾ Bushi-richu-to had no effects on plasma motilin-IS levels compared with the placebo group. It is difficult to elucidate the reason for this from the results of the present study. In general, somatostatin inhibited the secretion of motilin. Hence, our findings indicate that the stimulatory effect of Bushi-richu-to on somatostatin-IS secretion might be due, at least in part, to an effect on motilin-IS levels.

In the present study, Bushi-richu-to did not affect plasma motilin levels. However, in a previous study, Ninjin-to caused increases in motilin levels. Although both *Kampo* medicines contain the same gradients, motilin behavior after treatment of Bushi-richu-to is different from that of Ninjin-to. We believe the reasons are: the contents of both *Ginseng radix* and *Zingiberis rhizoma*, which have prokinetic effects, in Ninjin-to may be greater than those of Bushi-richu-to; Although Ninjin-to altered the levels of somatostatin, the levels were different from the changes in Bushi-richu-to in terms of the degree of increase; and *Aconiti tuber* inhibited motilin release from motilin-secreting cells. However, further studies are still needed. Also, Bushi-richu-to was found to have an effect on gut-regulated peptides in healthy subjects, so its effects on gut-regulated peptide levels in pathological conditions should be investigated.

It is thought that the chemical senses (taste and smell) of herbs often affect gastrointestinal action. Actually, it has been demonstrated that the taste and smell of food promote salivation, gastric acid secretion, pancreatic exocrine and endocrine secretions, and hormone (gastrin, and cholecystokinin *etc.*) secretions in addition to modulation of gastric motility/emptying.³⁹⁾ Since Bushi-richu-to has a specific fragrance and taste, it may affect plasma

gut-regulated peptide levels. However, it is difficult to demonstrate this from the results of the present study.

In conclusion, Bushi-richu-to ingestion results in increases in the plasma levels of CGRP-, substance P-, somatostatin-, and VIP-IS. We conclude that Bushi-richu-to may improve a peripheral and uncomfortable feeling of cold and gastrointestinal dysmotility by significantly increasing CGRP-, substance P-, somatostatin-, and VIP-IS levels in plasma.

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