Effects of Hange-koboku-to (Banxia-houpo-tang) on Neuropeptide Levels in Human Plasma and Saliva

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Hange-koboku-to (Banxia-houpo-tang), a Chinese herbal (Kampo) medicine, has been used for improvement of hoarse voice, something foreign body sensation in the throat and/or esophagus, and swallowing reflex, among other conditions. One of the mechanisms of the empirical effects is assumed to be due to local changes in neuropeptide levels locally. We investigated the effects of Hange-koboku-to on neuropeptides, calcitonin gene-related peptide (CGRP), substance P, somatostatin, and vasoactive intestinal peptide (VIP) in plasma and saliva, as well as on salivary secretion in healthy subjects. A single oral administration of Hange-koboku-to caused significant increases in substance P-immunoreactive substance (IS) (40 min) in plasma, and slightly increased in CGRP-IS and somatostatin-IS in plasma compared with placebo. In saliva neuropeptides, Hange-koboku-to caused significant increases in substance P-IS (20 min) and somatostatin-IS (40, 60 min), and a slight increase in VIP-IS. However, a single Hange-koboku-to stimulation did not have a significant effect of sialosis volume. These results seem to suggest that Hange-koboku-to improves hoarse voice, something foreign body sensation in the throat and esophagus, and swallowing reflex disorder, by stimulation of neuropeptidergic nerves locally.

Key words Hange-koboku-to (Banxia-houpo-tang); neuropeptide; substance P; saliva; Kampo medicine

Hange-koboku-to (Banxia-houpo-tang), a Chinese herbal (Kampo) medicine, is prepared from five crude herbs: Pinelliae Tuber, Hoelen, Magnoliae Cortex, Perillae Herba, and Zingiberis Rhizoma. This medicine has been empirically used for improvement of hoarse voice, something foreign body sensation in the throat and/or esophagus, and swallowing disorder in Parkinson’s disease, and other conditions. As for the empirical effects, some patients without organic pathological changes in the trachea and esophagus showed remarkable improvement. The pharmacological effects are assumed to be based on inhibition of the laryngeal reflex, improvement in swallowing reflex disorders, and moderating of anxiety and nervous tension.

Recently, a clinical study reported significant changes in saliva substance P levels after administration of Hange-koboku-to for 4 weeks in patients who had experienced at least one episode of aspiration pneumonia. As for this aspiration pneumonia, depletion of substance P in the pharynx caused disorders of the swallowing reflex. In other reports, capsaicin improved swallowing reflex in patients with cerebral thrombosis or dementia due to cerebral arterial sclerosis. Capsaicin stimulates capsaicin-sensitive afferent neurons which release calcitonin gene-related peptide (CGRP) and substance P from their nerve endings.

In general, a chronic lack of salivary secretion makes it difficult to swallow food. The salivary glands are supplied with nerve fibers that contain some neuropeptides such as CGRP and tachykinins (substance P, etc.). These peptides are involved in the secretion of saliva, and coexist in a population of sensory neurons in humans. CGRP, a powerful vasoactive substance, increases mucosal blood flow. Substance P is widely distributed in the central and peripheral divisions of the nervous system and gut enteroendocrine cells.

Exogenous CGRP stimulates gastric somatostatin release, and somatostatin release induced by capsaicin is mediated by CGRP in the stomach. The secretion mechanism suggests the existence of a functional linkage between CGRP and somatostatin. Vasoactive intestinal peptide (VIP) is widely distributed in the central and peripheral nervous systems as a brain-gut peptide. This peptide, like CGRP, has a vasodilatating effect in many vascular beds, and is an important neurotransmitter in the gastrointestinal nervous system.

There are only a few reports that have examined the temporal relationship among the neuropeptides (CGRP, substance P, somatostatin, and VIP) and sialosis volume with Hange-koboku-to stimulation. In this study, we examined the temporal effects of Hange-koboku-to on neuropeptide levels in plasma and saliva that were measured by a sensitive enzyme immunoassay, and salivary secretion measured by the Saxon test, in healthy subjects.

MATERIALS AND METHODS

Materials Hange-koboku-to (EK-16, lot 27A09), prepared as a 1.5-g dried powder extract of Pinelliae Tuber (6.0 g), Hoelen (5.0 g), Magnoliae Cortex (3.0 g), Perillae Herba (2.0 g), and Zingiberis Rhizoma (1.3 g), was kindly supplied by Kanebo (Tokyo, Japan). The placebo contained the EK-16 additives only.

Synthetic human CGRP and its fragment (8–37), substance P, somatostatin, and VIP were purchased from the Peptide Institute (Osaka, Japan). VIP fragment peptide (11–28) was supplied by Dr. H. Yajima of Kyoto University (Kyoto, Japan). Antisera to CGRP and VIP (A604/R1B) were purchased from Biogenesis (Poole, U.K.). Antiseria to substance P (RA-08-095) and somatostatin (RA-08-108) were purchased from Cambridge Research Biochemicals (Cambridge, U.K.). All other reagents were reagent grade and commercially available.

Subjects Five healthy male volunteers (nonsmokers), aged 24–29 years (median 26 years), participated in this study. Each subject received information about the scientific aim of the study and gave their written informed consent. The study was approved by the Ethics Committee of Oita Medical University. No subject received any medication for

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at least 1 month before the study.

**Study Schedule** Six grams of Hange-koboku-to was orally administered with water to the 5 subjects. Two weeks later, the same dose of placebo was given to the same 5 subjects. To measure neuropeptide levels, venous blood and saliva were taken before and 20—240 min after an administration of a test drug. Two months later, sialosis volume was measured in the same 5 subjects. Saliva was taken before and 60—240 min after an administration (five times). All subjects ate lunch at 11:45—12:00, and the study was carried out from 14:00 until 18:00.

**Sample Collections for Measuring Neuropeptide Levels** Blood withdrawn from a forearm vein was immediately placed in chilled tubes containing aprotinin (500 KIU/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). Unstimulated whole saliva specimens were collected by the spitting method according to Navazesh and Christensen.\(^{21}\) The subjects rinsed their mouth thoroughly with deionized water and rested for a few minutes before saliva collection began. After a 1-min practice collection, which was discarded, 2.5-ml of saliva was collected in a test tube containing aprotinin and EDTA over several minutes.

**Preparation of Plasma and Saliva Extract** The blood samples were centrifuged and then the plasma was diluted with 4% acetic acid (pH 4.0) and loaded onto Sep-Pak C18 cartridges (Millipore, MA, U.S.A.). The saliva samples were centrifuged, diluted with 4% acetic acid, centrifuged again, and then the supernatant was loaded onto Sep-Pak C18 cartridges. After washing with 4% acetic acid, peptides in plasma and saliva were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0), lyophilized, and stored at \(-40^\circ\)C. The recoveries of CGRP-, substance P-, somatostatin-, and VIP-IS\(^{25}\) as previously described. Human CGRP (8—37), substance P-, somatostatin-, and VIP were sensitive to detection limits of 0.084, 0.40, 1.0, and 0.10 fmol/well, respectively.

**Enzyme Immunoassay for Neuropeptides** Peptide levels in plasma and saliva were measured using enzyme immunoassays for CGRP,\(^{22}\) substance P,\(^{23}\) somatostatin,\(^{24}\) and VIP-IS\(^{25}\) as previously described. Human CGRP (8—37), substance P, somatostatin, and VIP (11—28) were conjugated with \(\beta\)-d-galactosidase (Boehringer Mannheim, Mannheim, Germany) with \(N\)-\((\varepsilon\)-maleimidocaproyloxy\)-succinimide.\(^{26}\) The assay was performed using a delayed addition method, and separation of bound and free antigen was performed on anti-rabbit IgG (55641) (ICN Pharmaceuticals, OH, U.S.A.)-coated immunoplates. The fluorescence intensity of the fluorescent product, 4-methylumbelliflorone, was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan). The enzyme immunoassays for CGRP, substance P, somatostatin, and VIP were sensitive to detection limits of 0.084, 0.40, 1.0, and 0.10 fmol/well, respectively.

**Measurement of Sialosis Volume** The amount of saliva was measured by the Saxon test, an oral equivalent of the Schirmer test.\(^{20}\) Two sterile absorbent cotton balls (No. 14, Kawamoto Houtai Zairyou, Osaka, Japan) and a polyethylene pouch were weighed. After swallowing to remove any existing oral fluid, saliva was collected by placing the two cotton balls onto the vestibule of the mouth for exactly 5 min. The subjects then expectorated the moist absorbent cotton balls into the polyethylene pouch. The amount of saliva produced for 5 min was determined by subtracting the original weight from the weight obtained after being placed in the mouth.

**Data Analysis** All values (neuropeptide levels and sialosis volumes) are expressed as relative values at each point to before taking test drug (0 min) and mean±S.D. (%). Comparisons of mean values were made by repeated measures two-way analysis of variance and paired t-test (placebo and Hange-koboku-to). A \(p<0.05\) was considered as indicative of statistical significance.

**RESULTS**

**Effect of Hange-koboku-to on Plasma Neuropeptide Levels** A plasma CGRP-IS level-time profile after administration of Hange-koboku-to is shown in Fig. 1A. Hange-koboku-to did not cause any significant change in plasma CGRP-IS levels. However, the participated subjects showed different degrees of increase in the Hange-koboku-to group, and the slight increases were observed between 20—60 min \([20 \text{ min (} p=0.067), 40 \text{ min (} p=0.066), 60 \text{ min (} p=0.063))\]. Figure 1B shows plasma substance P-IS levels after administration of Hange-koboku-to. Hange-koboku-to caused a significant increase only at 40 min \((p=0.021)\). Figure 1C shows plasma somatostatin-IS levels after an administration of Hange-koboku-to. There were no significant changes in plasma somatostatin-IS levels, although there was a slight increase at 60 min \((p=0.079)\). Hange-koboku-to had no significant effect on plasma VIP-IS levels (Fig. 1D). Plasma VIP-IS levels in both groups remained almost constant before and after administration.

**Effect of Hange-koboku-to on Saliva Neuropeptide Levels** Figure 2A shows saliva CGRP-IS levels after Hange-koboku-to stimulation increased slightly by about 1.2—1.4 fold compared to placebo at 60 min \((p=0.009)\) [120 min \((p=0.082)]\), and the changes occurred early on (Fig. 2B). The saliva somatostatin-IS level-time profile of Hange-koboku-to is shown in Fig. 2C. Somatostatin-IS levels increased significantly at 40 min \((p=0.045)\) and 60 min \((p=0.025)\), and slightly at 120 min \((p=0.078)\). Hange-koboku-to slightly increased VIP-IS levels in saliva between 40—90 min \([40 \text{ min (} p=0.054), 60 \text{ min (} p=0.071), 90 \text{ min (} p=0.056))\], but the effects were not significant (Fig. 2D).

**Effect of Hange-koboku-to on Sialosis Volume** The changes in sialosis volume after Hange-koboku-to administration are shown in Fig. 3. Hange-koboku-to had no significant effects on sialosis volume compared with placebo. The sialosis volume levels of placebo remained almost constant before and after administration, but the sialosis volume by Hange-koboku-to stimulation increased slightly by about 1.2—1.4 fold compared to placebo at 60 min \((p=0.051), 120 \text{ min (} p=0.083), \text{ and } 180 \text{ min (} p=0.081)\).

**DISCUSSION**

One of the swallowing reflex and salivary secretion regulatory factors is believed to cause local changes in neuropeptide levels. Hange-koboku-to has been used empirically for removal of something foreign body sensation in the throat and/or esophagus, and improvement of swallowing reflex disorders. These effects are assumed to be based on changes in
neuropeptide levels.4) CGRP functions as a salivary secretion and gastrointestinal protective factor.12,27) In the esophagus, CGRP-immunoreactive axons are distributed to nerve fibers. The lower esophagus is an important site for the swallowing reflex, and CGRP affects the lower esophageal muscle.28) In the Hange-koboku-to administration group, CGRP levels increased to different degrees among the subjects so significant effects on neuropeptide levels could not be found. The increase ratio between plasma and saliva showed the almost same degree of increase. The factors that caused the different degree of increase among the subjects are unknown.
CGRP coexists with tachykinins, which include substance P and neurokinin A, among others, in sensory afferent neurons of the gastrointestinal mucosa, and is released with acetylcholine in response to depolarizing stimuli in the gastrointestinal nervous system.\(^\text{(29,30)}\) In this study, Hange-koboku-to significantly raised substance P levels in both plasma and saliva, and the changes in substance P occurred early on. Plasma substance P levels might be linked with changes in saliva substance P levels, so changes in the saliva might be an index which reflects the pharmacological effects in the throat and esophagus. As for regulation of the swallowing reflex, which is mediated by endogenous substance P released from vagal sensory nerves in the pharynx and upper airway, substance P plays an important role as a regulator.\(^\text{(31)}\)

Some ingredients of Hange-koboku-to might be involved in the changes in substance P. Magnorol and konokiol from Magnoliae Cortex, and perillaldehyde from Perillae Herba have been found to have inhibitory actions on the central nervous system, so the authors speculated that the actions might be related to inhibition of the laryngeal reflex.\(^\text{(32,33)}\) Zingiberis Rhizoma, an ingredient of Hange-koboku-to, contains 6-gingerol and 6-shogaol as its main bioactive compounds. These compounds have vanilloid structures, and act as capsaicin-like stimulation of vanilloid receptors. In a previous study, we reported that Zingiberis Rhizoma and a Kampo medicine compounds have vanilloid structures, and act as capsaicin-like stimulation of vanilloid receptors. In a previous study, we reported that Zingiberis Rhizoma and a Kampo medicine contains it, Hange-shashin-to (Banxia-xiexin-tang), raised plasma CGRP and substance P levels.\(^\text{(34)}\)

A functional linkage between somatostatin and CGRP affects regulation of the secretion of each neuropeptide.\(^\text{(18)}\) Hange-koboku-to caused a significant increase in somatostatin levels in saliva. The changes in saliva somatostatin levels were 3 fold greater than those of plasma. With respect to the relationship between Hange-koboku-to stimulation and somatostatin, the monitoring of saliva somatostatin levels may be able to predict the pharmacological effects of Hange-koboku-to.

VIP, like CGRP, is an important vasodilating factor and neurotransmitter in the brain and gastrointestinal nervous system. The secretion of VIP is related to an increase in blood flow in internal organs. Hange-koboku-to did not significantly increase VIP levels in either plasma or saliva. However, saliva VIP levels increased slightly between 40 min and 90 min and the changes were as large as those of placebo. The mechanism behind the changes in VIP-IS levels caused by Hange-koboku-to at some subjects is unknown.

A continuously low sialosis volume tends to result in a dry mouth and difficulty swallowing food. Sjögren's syndrome is an autoimmune disease characterized by inflammation in the exocorial glands, especially in the salivary glands.\(^\text{(35)}\) Most Sjögren's syndrome patients suffer from enduring xerostomia (dry mouth), and the condition causes swallowing reflex disorders.\(^\text{(36)}\) The salivary glands are supplied with nerve fibers that contain the neuropeptides CGRP and substance P. Anethole trithione and pilocarpine hydrochloride, which affect salivary secretion, have been reported to raise CGRP and substance P levels in human saliva.\(^\text{(37—39)}\) However, Hange-koboku-to had no significant effects on sialosis volume in this study. We examined the neuropeptide levels after a single administration of Hange-koboku-to. Repeated administration of Hange-koboku-to might reveal changes in sialosis volume and the relations among the neuropeptides.

We examined the temporal effects of Hange-koboku-to on neuropeptides after a single administration in healthy subjects. Those neuropeptidergic effects should be necessary to examine on the pathological conditions and at the local throat and esophagus. The results suggest that one mechanism by which Hange-koboku-to improves hoarse voice, something foreign body sensation in the throat and esophagus, and swallowing reflex, is by local stimulation of neuropeptidergic nerves.

**REFERENCES AND NOTES**

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