Effects of bakumondoto on neuropeptide levels in human saliva and plasma

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Abstract

Bakumondoto is empirically used to treat to dry mouth (xerostomia) associated with salivary gland dysfunction. Salivary glands are supplied with nerve fibers that contain neuropeptides, such as substance P, calcitonin gene-related peptide (CGRP), and vasoactive intestinal polypeptide (VIP). These neuropeptides are important modulators of salivation. We studied the effect of bakumondoto on substance P-, CGRP-, VIP-like immunoreactive substances (IS) in saliva and plasma taken from healthy subjects. Bakumondoto (18.0 g) or placebo was orally administered to five healthy males. Saliva and blood samples were taken before, and at 20-240 min after administration, followed by the extracting procedure, and submitted to a highly sensitive enzyme immunoassay system for neuropeptides. A single oral administration of bakumondoto caused significant increases in saliva substance P- and CGRP-IS levels compared with placebo. And this medicine tended to raise saliva volume. In this study, we concluded bakumondoto might affect substance P and CGRP locally in the salivary gland and the elevated levels of these neuropeptides might stimulate production of saliva. Also, the monitoring of saliva and plasma neuropeptide levels might be able to predict the pharmacologic effects of bakumondoto.

Key words  bakumondoto, substance P, calcitonin gene-related peptide (CGRP), xerostomia, saliva secretion.

Introduction

Saliva is a major protective factor in the oral cavity, providing protection for oral hard and soft tissues and support for critical oral functions. Xerostomia (dry mouth) is associated with a variety of diseases, but can also be caused by Sjögren’s syndrome and numerous drugs. The loss of the antimicrobial, buffering, remineralizing, and cleansing properties of saliva when salivary output is reduced might lead to rapid and severe caries. Other complications of salivary hypofunction include an increase in oral infections, mucosal pain and friability, difficulties with chewing, swallowing and speaking.1,2)

Bakumondoto is a traditional Chinese meditational prescription consisting of six herbs (Ophiopogon Tuber, Pinellia Tuber, Zizyphi Fructus, Glycyrrhiza Radix, Ginseng Radix, and Oryzae Semen). This formula is empirically used for the treatment of bronchitis, bronchial asthma, and hacking cough. Several studies suggest that it could be effective in relieving oral symptoms, including dry mouth, halitosis, and glossodynia. In clinical trial subjects complaining of dryness of the mouth and throat, administration of bakumondoto resulted in significant improvement in subjective and objective symptoms.3-5) The good efficacy also has been reported for patients with Sjögren’s syndrome and xerostomia.6)

The functions of the salivary glands are controlled by the autonomic nervous system and influence by the sensory nervous system. It has shown that the salivary

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glands are supplied with nerve fibers that contain some neuropeptides such as tachykinin (substance P etc.), calcitonin gene-related peptide (CGRP), and vasoactive intestinal polypeptide (VIP). These peptides are involved in the secretion of saliva, and regulate the releases of some neurotransmitter or neuropeptides. Substance P, a member of the tachykinin family, induces water and electrolyte secretion in the salivary gland. CGRP evokes both salivary secretion and vasodilation in the salivary gland. VIP evokes the release of a small volume of saliva that is rich in protein.

At present, there are also few reports that have examined the temporal relationship among the neuropeptides and saliva volume with bakumondoto stimulation. In this study, we examined the effects of bakumondoto on neuropeptide levels in saliva and plasma that were measured by a sensitive enzyme immunoassay (EIA), and saliva volume measured by the Saxon test, in healthy subjects.

Materials and Methods

Materials: Bakumondoto (TJ-29, lot: 25105132) prepared as a dried powder extract of Ophiopogon Tuber (10.0 g), Pinellia Tuber (5.0 g), Zizyphi Fructus (3.0 g), Glycyrrhiza Radix (2.0 g), Ginseng Radix (2.0 g), and Oryzae Semen (5.0 g), was purchased from Tsumura and Co. (Tokyo, Japan). A mixture of maltose and lactose (1:1) was used as placebo. Synthetic human VIP, CGRP and its fragment (8-37), substance P, were purchased from the Peptide Institute (Osaka, Japan). The VIP fragment was supplied by Professor H. Yajima (Kyoto University, Kyoto, Japan). Antiserum to VIP (A604/R1B) and CGRP (CA1132) were purchased from Biogenesis (Poole, UK) from Peninsula Laboratories (San Carlos, CA), from Yanaihara Institute (Shizuoka, Japan) and substance P (RA-08-095) from Cambridge Research Biochemicals (Cambridge, UK). Goat affinity-purified antibody to rabbit IgG (whole molecule) (55641) was purchased from ICN Pharmaceuticals (Aurora, OH, USA). All other reagents were of analytical reagent grade from commercial sources.

Subjects: Five healthy male volunteers (nonsmokers), aged 23-30 years (median 26 years), weighing 55-68 kg (median weight 62 kg), 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 proceeding the test and no stimulator of gastrointestinal motility was administered to any subjects during the study.

Study protocol: This was a two-way crossover (bakumondoto and placebo) study. Bakumondoto (18.0 g) or placebo was orally administered with 100 ml water. Each subject was administered these drugs with an interval of four weeks. Saliva was sampled before and at 20, 40, 60, 90, 120, 180, and 240 min after administration of the test substances. Venous blood samples (10 ml) from a forearm vein were also taken at each time interval. The study was carried out from 14:00 to 18:00.

Measurement of whole saliva volume: The amount of saliva was measured by the Saxon test. Two sterile 5-g absorbent cottons were weighed. After swallowing, saliva was collected by setting the two cottons onto the vestibule of the mouth for 5 min. The subjects then expectorated the moist absorbent cottons onto a plastic tray. The cottons were weighed on electron balance (AE240: Mettler Instrumente, Switzerland), accurate to 10⁻³g. The amount of saliva produced in 5 min was determined by subtracting the original weight from the final weight of the cottons.

Sample collection for measuring neuropeptide levels: Blood withdrawn from a forearm vein was immediately placed in chilled tubes containing aprotinin (500-kallikrein inhibitor units ml) and ethylenediaminetetra-acetic acid (EDTA) (1.2 mg/ml). Resting whole saliva specimens were collected by the spitting method according to Navazesh and Christensen and Nagano et al. Subjects rinsed thoroughly with deionized water and rested for 1 min before saliva collection. After a 30-s practice collection, which was discarded, saliva (2.5 ml) was collected in a test tube (Nunc-Immuno Tube Minisorp 75 × 12; InterMed, Denmark) over 5 min.
Preparation of saliva and plasma extracts: 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 Corp., Milford, USA). The saliva sample was 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 the saliva and plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0), lyophilized, and stored at −40°C. The recovery of plasma and saliva CGRP-, substance P-, VIP-, and somatostatin-IS was >90% with this extraction procedure.

EIAs of, substance P, CGRP, and VIP: EIAs for substance P,18, CGRP,19, and VIP-IS20 were performed as previously described, by a delayed-addition method.21 Separation of bound and free antigen was performed on an 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)-succimide according to the method Kitagawa et al.22 The EIAs for VIP, CGRP, and substance P was specific and highly sensitive to detection limits of 1.00, 0.08 and 0.40 fmol/well, respectively. The area under the saliva and plasma concentration-time curve from zero to 240 min was also calculated.

Statistical analysis: All values are expressed as the mean ± S.D. Comparisons between groups was analyzed with analysis of variance for repeated measure. Paired observations were analyzed using the Mann Whitney U-test. Value of p < 0.01 or p < 0.05 was considered to represent a statistically significant difference.

Results

Effect of bakumondoto on saliva neuropeptide levels: Figure 1A shows saliva substance P-IS level-time profile after administration of bakumondoto to healthy subjects. Bakumondoto significantly increased substance P-IS at 40 min (37.8 ± 14.7 pg/ml, p=0.0317) compared with the response of the placebo group (23.5 ± 10.2 pg/ml). Figure 1B shows saliva CGRP-IS levels after administration of bakumondoto to healthy subjects. Bakumondoto significantly increased CGRP-IS at 90 min (65.5 ± 34.4 pg/ml, p=0.0079) compared with placebo (24.8 ± 4.5 pg/ml). The saliva VIP-IS level-time profile after administration of bakumondoto to healthy subjects is shown in Fig. 1C. Bakumondoto did not change levels of VIP.

Effect of bakumondoto on plasma neuropeptide levels: Figure 2A shows plasma substance P-IS level-time profile after administration of bakumondoto to healthy subjects. Bakumondoto significantly increased substance P-IS at 90 min (34.1 ± 14.0 pg/ml, p=0.0127) compared with the response of the placebo group (23.3 ± 2.8 pg/ml). Figure 2B shows plasma CGRP-IS levels after administration of bakumondoto to healthy subjects. Bakumondoto did not change CGRP-IS levels in plasma. The plasma VIP-IS level-time profile after treatment of bakumondoto to healthy subjects is shown in Fig. 2C. Bakumondoto did not change levels of VIP.

Relationship among saliva and plasma levels of substance P after treatment of bakumondoto: A simple linear regression of the area under saliva and plasma concentration-time curve from zero to 240 min after treatment of test drugs was in shown Fig. 3. Significant correlation (r= 0.78) was found between the saliva and plasma substance P levels.

Effect of bakumondoto on saliva volume: The changes in whole saliva volume after bakumondoto administration are shown in Fig. 4. The saliva volume of placebo remained almost constant before and after administration, but the saliva volume by bakumondoto stimulation increased by 137–, 126–, and 133 % at 20, 40, and 60 min, respectively.

A simple linear regression of the increase in saliva volume with saliva substance P levels at each sampling points after treatment of test drugs in shown Fig. 5. Correlation (r = 0.66) was found between the saliva volume increase and saliva substance P levels.
Fig. 1. Changes of saliva substance P (A)-, CGRP (B)-, somatostatin-(C)-, and VIP (D)-IS levels after a single administration of bakumondoto 18.0 g (●) or placebo (○) Each point represents the mean ± S.D. of five healthy subjects. **p < 0.01 and *p < 0.05 compared with placebo.
Fig. 2. Changes of plasma substance P (A)-, CGRP (B)-, somatostatin- (C)-, and VIP (D)-IS levels after a single administration of bakumondoto 18.0 g (●) or placebo (○). Each point represents the mean ± S.D. of five healthy subjects. *p < 0.05 compared with placebo.
Fig. 3. Relationship between saliva and plasma substance P area under the curve (AUC) from 0 to 240 min after oral administration of bakumondoto (18.0 g) (■) or placebo (○) to 5 healthy subjects. n=10

Fig. 4. Changes of saliva volume after a single administration of bakumondoto (18.0 g) (■) or placebo (○)

Fig. 5. Relationship between the changes of saliva volume and mean levels of substance P in saliva at each times after oral administration of bakumondoto (18.0 g) or placebo to 5 healthy subjects. n=16
Discussion

One of the salivary secretion regulatory factors is believed to cause local changes in neuropeptide levels. Salivary secretion in Sjögren’s syndrome and medication of fractionated irradiation decrease and local neuropeptide levels such as substance P are changed compared with healthy human. In the present study, we examined whether the pharmacologic effects of bakumondoto are based on local changes in neuropeptide levels.

Bakumondoto significantly raised substance P levels, and its changes showed the same action in comparison with saliva volume, except that the peak time of the plasma substance P levels was later.

Substance P is mainly distributed in salivary parotid and submandibular glands. This peptide has a potent alogic activity. The experiment applied to cultured cells revealed that bakumondoto stimulated the salivary glands. Saliva volume increased, synchronously the increases in levels of salivary substance P after a single treatment of bakumondoto. It is suggested that bakumondoto might secrete substance P accompanied by direct stimulation of sensory neurons or other mechanisms (ex. muscarinic receptor etc.). In addition, plasma substance P levels were correlated with the changes in saliva after administration of test drugs. The results indicate that measuring plasma levels of peptides is useful for assessing the kinetics of local substance P and the pharmacological effects of bakumondoto.

CGRP-containing nerves were mainly localized around blood vessels and ducts in salivary glands. CGRP also increases the blood flow in the salivary glands and enhances the salivary secretion caused by substance P and acetylcholine. In this study, bakumondoto significantly raised CGRP levels in saliva. Bakumondoto might act in the salivary glands and part of its action might be closely related to changes of CGRP levels in saliva. On the other hands, changes of saliva CGRP levels showed a delayed action in comparison with those of saliva volume after administration of bakumondoto. It is difficult to elucidate its reason from the results of the present study. It is of interest to report that: the sympathetic ganglion of the rat contains CGRP confined to neurons different from substance P. CGRP also causes an increase of the salivary secretion smaller than substance P. Bakumondoto did not change plasma levels of CGRP compared with placebo. CGRP in plasma is mainly from gut and blood vessels. Bakumondoto might have localized effect in salivary glands.

VIP is a potent vasodilator in the human submandibular gland. VIP relatively produces small amounts of saliva secretion, and enhances the salivary secretion caused by other neuropeptides as well as CGRP. In our results, bakumondoto did not alter VIP levels in neither saliva nor plasma. VIP might be not related with the pharmacologic effect of bakumondoto.

Sjögren’s syndrome is characterized by a generalized chronic inflammatory disease of the exocrine glands including salivary glands. Patients with Sjögren’s syndrome show signs and symptoms of decreased salivation, leading to dry mouth with destruction of the exocrine glands. There are many systemic agents that are capable of stimulating salivary output. Western medicine with extensive clinical evidence is pilocarpine hydrochloride or anethole trithione. These drugs have reported to raise CGRP and substance P levels in human saliva and saliva volume. In oriental herbal therapy, Kampo medicines such as hangekobokuto and bakumondoto have alogic effects, and they sometimes produce remarkable efficacy for salivary gland dysfunctions. In clinical study, Nishizawa et al. reported that bakumondoto improved symptoms of deceased salivation by stimulation of muscarinic receptors. Hangekobokuto, which is mainly used for improvement of swallowing disorder, has reported to raise substance P levels in human saliva and plasma. In our study, the stimulatory effect of bakumondoto on saliva secretion might be due, at least in part, to an effect on substance P and CGRP by stimulation of muscarinic receptor or other mechanisms. However, we examined the neuropeptide levels after a single administration of bakumondoto. Repeated administration of bakumondoto might reveal changes in saliva volume and the relations among neuropeptides.

In conclusions, bakumondoto increases in the saliva levels of substance P and CGRP levels, and its increases correlates with enhanced salivary volume in humans. Bakumondoto, by increasing substance P and CGRP release might contribute to the improvement of dry mouth.
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References

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