Mechanism of Salivary Secretion Enhancement by Byakkokaninjinto

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Byakkokaninjinto (BN) is a Kampo preparation used for the treatment of xerostomia induced by drug, aging, Sjogren syndrome, etc. The mechanism for BN to induce salivary secretion has not been made clear. In this study, various rat thirst models were prepared using muscarinic receptor blockers, such as 4-diphenylethoxy-N-methylpiperidine (4-DAMP) and atropine, or adrenoceptor blockers, such as phentolamine and propranolol, in order to investigate the efficiency of BN. When BN was orally administered to the rats in the dose range of 100 to 300 mg/kg, the salivary secretion increased in a dose-dependent manner. The suppression of salivary secretion induced by phentolamine, atropine, and 4-DAMP was recovered by the additional treatment of BN. Interestingly, BN treatment increased the expression of aquaporin 5 in rats, which is known to regulate salivary secretion from the submandibular gland. These results suggested that BN increased the expression of aquaporin 5 through activation of muscarinic M3 receptor and enhanced salivary secretion.

Key words Byakkokaninjinto; Xerostomia; salivary secretion; muscarinic M3 receptor; aquaporin 5

Xerostomia is a symptom occurring with certain diseases, such as Sjogren’s syndrome,1) or an adverse reaction to some drugs such as anticholinergic agents. An increasing number of patients are experiencing thirst as an adverse reaction to anticholinergic agents used frequently for the treatment of urinary incontinence or frequent urination. This tendency is considered to continue in the rapidly aging society.2—4) Xerostomia produces serious negative influences on patient’s quality of life, affecting the nutritional status, speech, taste, rostromia produce serious negative influences on patient’s taste, etc. These influences result in delay of patient recovery.5,6) Some traditional herbal medicines are used for the treatment of xerostomia, but among those, Byakkokaninjinto (BN) is different from the others containing ginseng root and Glycyrrhiza root, including saponin. BN is used frequently for the treatment of xerostomia in Japanese elderly patients.7) In the BN preparation used in this study, TJ-34 of Tsumura, the quality and quantity of each of the components are standardized. BN contains the extracts of 5 medicinal plants, Gypsum fibrosum (gypsum), Anemarrhena rhizoma (rhizome of Liliaceae Anemarrhena asphodeloides Bunge), Glycyrrhiza radix (radix of Leguminosae Glycyrrhiza uralensis Fisch), Ginseng radix (radix of Araliaceae Panax ginseng C. A. Mey), Oryzae fructus (grain of Gramineae Oryza satival). We previously reported the components contained in the BN methanol extract by analyzing the three-dimensional HPLC fingerprint.8) It was reported that saponin enhances cell membrane permeability and facilitates incorporation of various kinds of cell-activating factors.9) However, the inhibitory effect of BN on xerostomia has not been fully elucidated.

Salivary secretion is promoted by acetylcholine and norepinephrine released from the nerve endings.10) Excitation of the parasympathetic nerve induces secretion of serous saliva by stimulating the muscarinic receptors of the salivary gland,11) whereas excitation of the sympathetic nerve induces secretion of mucous saliva by stimulating the adrenoceptor of the salivary gland. In addition, aquaporins have been reported to be implicated in the mechanism of salivary secretion.10) Aquaporin (AQP) is a six-transmembrane receptor family and 13 subtypes (AQP 0—12) have been identified in various organs.12) Five subtypes of aquaporin, i.e., AQP-1, AQP-3, AQP-4, AQP-5 and AQP-8 have been identified in the salivary gland. AQP-3, AQP-5 and AQP-8 are found in the acinar cells of humans and rats. AQP-5 is usually found on the luminal-side of the membrane of humans and rats. AQP-3 is expressed on the vascular-side membranes in humans, and AQP-8 is expressed on the vascular-side membranes in rats. AQP acts as a pipe connecting between the vascular and luminal sides of the cell and is involved in transportation of water. The amount of water transported by AQP is considered to account for about 60% of the total amount of saliva.12,13) In the present study, some rat thirst models were prepared by using a muscarinic receptor-blocking agent or an adrenoceptor-blocking agent, and the mechanical aspect of the BN effect on salivary secretion was investigated. In addition, the BN effect on expression of AQP-5 was also investigated.

MATERIALS AND METHODS

Experimental Animals Male Wistar rats (aged 7—8 weeks) were purchased from Saitama Experimental Animals Co., Ltd. (Saitama, Japan). The animals were provided with solid food and tap water ad libitum. All animal experiments were carried out according to the guidelines of the Committee of Animal Care and Welfare of Showa University.

Reagents BN (Tsumura’s BN Extract Preparation, TJ-34, Lot No. 240034010, manufactured by Tsumura & Co., Tokyo, Japan) is a powdered extract preparation made from five medicinal plants: JP Gypsum fibrosum (gypsum, 15 g), JP Anemarrhena rhizoma (rhizome of Liliaceae Anemarrhena asphodeloides Bunge, 5 g), JP Glycyrrhiza radix (radix of Leguminosae Glycyrrhiza uralensis Fisch, 2 g), JP Ginseng radix (radix of Araliaceae Panax ginseng C. A. Mey, 1.5 g), Oryzae fructus (grain of Gramineae Oryza sati-
JP stands for the Japanese Pharmacopoeia XVth Edition. Acetylcholine chloride was purchased from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). EDTA, sodium chloride, 2-mercaptoethanol, polyoxyethylene (20), sorbitan monolaurate (Tween 20), glycine, methanol, sodium dodecyl sulfate (SDS) and atropine sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Phentolamine, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) and urethane were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Propranolol hydrochloride was purchased from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). Ultra Pure Tris was purchased from Invitrogen Japan K.K. (Tokyo, Japan).

Analysis of Three-Dimensional HPLC Fingerprint of BN
BN (1.0 g) was extracted with methanol (20 ml) under ultrasonication for 30 min. The mixture was filtered and then subjected to HPLC analysis. The three-dimensional HPLC chart of the methanol solution of BN is shown in Fig. 1.

Preparation of Xerostomia Model and Measurement of Saliva Secretion in Rats
Each male Wistar rat was anesthetized by intraperitoneal administration of urethane (1.3 g/kg) and placed in a supine position after confirming the loss of righting reflex. Physiological saline or BN was orally administered, followed by insertion of a cotton ball under the tongue to collect the saliva secreted. The cotton ball was exchanged with a new one every 30 min. The saliva secreted was collected for 150 min after the oral administration and the cotton balls were weighed. Four types of thirst models were prepared by intravenous administration of each of the following drugs: 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) and atropine, which are muscarinic receptor blockers, and phentolamine and propranolol, which are adrenoceptor blockers. Each drug was intravenously administered at a dose level of 1 mg/kg. Subsequently, BN was orally administered at 20 min after intravenous administration of each of the above drugs, and salivary secretion was measured for 30 min. The mass of saliva secreted was calculated from the difference between the initial and final cotton ball weights.

Western Blotting Analysis of AQP-5
The amount of AQP-5 protein was determined using the anti-AQP-5 antibody (Alpha Diagnostic International Inc., San Antonio, Texas, U.S.A.). Acetylcholine (4 mg/kg) was orally administered as an aquaporin expression-inducing positive control. At 30 min after administration of BN or acetylcholine, the bilateral submandibular glands were isolated by cervical incision and stored in 1 ml of the 8 M urea buffer (8 M urea, 50 mM Tris HCl (pH 8), 1 mM dithithreitol and 1 mM EDTA)) for 1 h. The isolated submandibular glands were minced and homogenized with a homogenizer (Polytron). The homogenate was centrifuged at 4 °C and 10000×g for 30 min. After discarding the precipitates, the supernatant was subjected to ultracentrifugation at 4 °C and 40000×g for 10 min. After discarding the supernatant, the precipitates were dissolved by adding 200 μl of a fresh 8 M urea buffer. After measuring the concentration of the precipitates, western blot analysis was carried out to determine the AQP-5 expression level. The samples were denatured at 100 °C for 5 min and cooled rapidly in ice-water. Twenty-five micrograms of protein was ap-

Fig. 1. Three-Dimensional HPLC Chart of Methanol Solution of BN
HPLC operating conditions were as follows: HPLC system (LC10AD, Shimadzu Corporation, Kyoto, Japan), Column (TSK-GEL ODS-80Ts (4.6 mm in inside diameter×250 mm in length)), Mobile phase (Phase A: 0.05 M AcONH₄ (pH 3.6), Phase B: CH₃CN), Gradient condition (Linear gradient from “90% Mobile Phase A and 10% Mobile Phase B” to “0% Mobile Phase A and 100% Mobile Phase B” for 60 min (maintenance at “100% Mobile Phase B” for 20 min), Flow rate (1.0 ml/min). The eluted substrates from the column were monitored and the three-dimensional data were processed by a diode array detector (SPD-M 10 Asp. Shimadzu Corporation).
plied to 4—20% SDS polyacrylamide gradient gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan, P AG MINI “Daiichi” 4/20 (13 W)) and subjected to electrophoresis at 70 mA for 90—110 min. The separated proteins were transferred to nitrocellulose membrane and blocked with the blocking solution (10% nonfat mild, 0.05% Tween 20 and 0.5% sodium azide in PBS) at 4 °C overnight. The blocked membrane was washed twice with the washing buffer (0.05% Tween 20 in PBS) for 5 min, and then the membrane was incubated with the 1000 times diluted anti-AQP-5 polyclonal antibody for 1 h at room temperature. The anti-AQP-5 antibody (in 0.05% sodium azide solution) was added at a concentration of 1 μl/ml, followed by washing with the washing buffer for 15 min and then washing three times for 5 min each time. The membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-labeled anti-rabbit IgG goat polyclonal antibody as the secondary antibody diluted 10000 times. Then, the membrane was washed with the washing buffer for 15 min and further washed three times for 5 min each time. The immune complexes were detected with ECL Kit (ECL Advance Western Blotting Detection, Amersham Biosciences Co., Ltd., Tokyo, Japan). The blot density was analyzed using image analysis software Image Hyper II.

Statistical Analysis The results are expressed as the mean±S.E.M. of n observations for each experiment. Statistical analyses were performed with the Bonferroni/Dunn procedure following ANOVA or the Mann–Whitney test. Differences between means were considered significant at p<0.05.

RESULTS

Salivary Secretion Induced by BN Salivary secretion was compared between the BN-treated rats and the physiological saline-treated rats. The salivary secretion was increased by the BN in a dose-dependent manner in the dose range of 100 to 300 mg/kg, and the maximum secretion was seen at 300 mg/kg (Fig. 2). The salivary secretion reached a peak at 30 min after administration of BN at 300 mg/kg and thereafter decreased to the baseline level (Fig. 3).

Effect of BN on Rat Salivary Secretion in Rats Treated with Propranolol When propranolol hydrochloride, a β-adrenoceptor blocker, was intravenously administered at 1 mg/kg, no changes or tendency to decrease were seen in salivary secretion. Additional oral administration of BN (300 mg/kg) increased the salivary secretion (Fig. 4).

Effect of BN on Rat Salivary Secretion Suppressed by Phentolamine When phentolamine, an α adrenoceptor blocker, was intravenously administered at 1 mg/kg, rat salivary secretion was suppressed. The salivary secretion suppressed by the phentolamine treatment was significant recovered and rather enhanced by additional oral administration of BN (300 mg/kg) (Fig. 5).

Effect of BN on Rat Salivary Secretion Suppressed by 4-DAMP When 4-DAMP, a selective muscarinic M3 recep-

Fig. 2. Effect of BN on Salivary Secretion of Rats
The rats were orally administered physiological saline or BN (100, 200, 300, 600 mg/kg) and the amount of saliva secreted was determined by collecting it with cotton balls for 30 min after administration. ∗p<0.05 vs. physiological saline (n=10).

Fig. 3. Time-Course of Salivary Secretion after BN Administration of BN of Rats
The rats were orally administered physiological saline or BN (300 mg/kg) and the amount of saliva was determined for 30 min. Each open circle and closed circle indicates the data of physiological saline and BN respectively. ∗p<0.05 vs. physiological saline (n=10).

Fig. 4. Effect of BN Administered Subsequently to Propranolol
The rats were intravenously administered physiological saline (control), propranolol (1 mg/kg). BN (300 mg/kg) was orally administered at 20 min after administration of propranolol. The amount of saliva secretion was determined for 30 min after administration of each drug. ∗p<0.05 vs. physiological saline and #p<0.05 vs. phentolamine (n=5).

Fig. 5. Effect of BN on Phentolamine-Induced Salivary Diminution
The rats were intravenously administered physiological saline (control) and phentolamine (1 mg/kg). BN (300 mg/kg) was orally administered at 20 min after administration of phentolamine. The amount of saliva secretion was determined for 30 min after administration of each drug. ∗p<0.05 vs. physiological saline and +p<0.05 vs. phentolamine (n=5).
tor blocker, was intravenously administered at 1 mg/kg, salivary secretion was strongly suppressed. When the salivary secretion accumulated during 1 h was compared, 300 mg/kg of BN recovered the secretion suppressed by the 4-DAMP (Fig. 6).

Effect of BN on Rat Salivary Secretion Suppressed by Atropine  When atropine, an anti-muscarinic agent, was intravenously administered at 1 mg/kg, salivary secretion was suppressed almost completely. The salivary secretion suppressed by the atropine treatment was significant recovered by additional oral administration of BN (300 mg/kg) (Fig. 7).

Induction of AQP-5 Expression by Acetylcholine and BN  AQP-5 is a protein with a molecular weight of 27 kDa. It is known that acetylcholine induces AQP-5 expression. Expression of AQP-5 was observed even in the submandibular glands of the control rats, and oral administration of acetylcholine (4 mg/kg) increased the AQP-5 expression, and also oral administration of BN (300 mg/kg) increased the AQP-5 expression (Fig. 8).

DISCUSSION

In the present study, some rat xerostomia models were prepared using muscarinic receptor-blocking agents or adrenoceptor-blocking agents, and the effect of BN was investigated. Secretion of saliva was increased after oral administration of BN in a dose-dependent manner in the dose range of 100—300 mg/kg, showing the maximum effect at 300 mg/kg. When the dose was increased to 600 mg/kg, however, the salivary secretion decreased to the baseline level. The reason why the salivary secretion was decreased by increasing the dose of BN to 600 mg is not clear, it is suggested that BN may contain some suppressing components to salivary secretion.

It is known that salivary secretion from the gland (the sublingual, submandibular and parotid gland) is regulated by the parasympathetic nerve but the submandibular and parotid glands are also controlled by the sympathetic nervous system. Hyperdepolarization-activated 

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\text{\textit{c}}1\text{ adrenoceptor in saliva cyner cell secretes mainly saliva containing electrolytes and H}_2\text{O. Stimulation of } \beta2 \text{ adrenoceptor induces depolarization and secretes saliva containing organic elements such as amylase and mucin although it’s only a small amount of secretion. In the present study, administration of BN (300 mg/kg) significantly restored salivary secretion suppressed by administration of phentolamine (1 mg/kg), an } \alpha \text{ adrenoceptor blocker. Propranolol (1 mg/kg), } \beta1/\beta2 \text{ non-selective adrenoceptor blocker indicated a tendency to slightly suppress salivary secretion. BN recovered the adrenoceptor blockers-induced suppression of the salivary secretion and enhanced the salivary secretion. These results suggested that a part of the mechanisms of salivary secretion by BN might be related with the adrenoceptor.}

When 4-DAMP; a selective muscarinic M3 receptor blocker, was intravenously administered at 1 mg/kg, salivary secretion was strongly suppressed. Oral administration of BN recovered the secretion suppressed with 4-DAMP during 1 h. Additionally, atropine-induced salivary depression was also recovered with BN. These results suggest that the salivary secretion enhanced with BN is mediated by the muscarinic M3 receptor.

It was reported that AQP-3, AQP-5 and AQP-8 are found in the salivary glands of rats and humans and that they play a role in the transportation of water between the vascular and luminal sides of the cell. When acetylcholine (4 mg/kg) was orally administered, salivary secretion was enhanced markedly and at the same time an increase in the expression of AQP-5 was observed in the membrane fraction of the submandibular gland. Interestingly, also when BN (300 mg/kg) was orally administered, an increase of the AQP-5 expression was induced. These results suggest that the pro-
motion of transferring of AQP-5 to the membrane is involved at least partly as the mechanism of salivary secretion-enhancing effect of BN. Expression of AQP-3/AQP-8 was also investigated (data not shown), but there was no evidence to suggest enhanced expression of these proteins. In other studies,13,15) the membrane fractions were used in an amount ranging from 100 to 300 μg for investigating the expression of AQP-3/AQP-8, but in the present study only about 10 μg was used. The reason for the failure to detect expression of AQP-3/AQP-8 might be an insufficient amount of protein. The fact that the expression of AQP-5 was detected in the submandibular gland, in spite of the small amount of the protein used, suggests that the AQP-5 expression is of greater importance when compared with the other aquaporins in rats.

These results obtained in this study suggest that the action mechanism of BN in the induction of salivary secretion involves a phospholipase C-mediated increase in calcium ion concentration and calmodulin activation as a result of stimulating the muscarinic M3 receptor, followed by transfer of AQP-5 to the luminal membrane.16) A new action mechanism of BN related to the expression of AQP-5 was discovered, but it remains unclear whether the active components of BN actually bind to the muscarinic receptor. Further investigations are therefore needed.

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REFERENCES