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The effects on pilocarpine-induced saliva secretion by a hot aqueous extract of Byakko-ka-ninjin-to (BN), its constituents, rhizomes of *Anemarrhena asphodeloides*, three saponins (pseudoprotosaponin-AIII (An-S-1), proto-timosaponin-AIII (An-S-2) and timosaponin-AIII (An-S-3)) and calcium were examined in streptozocin (STZ)-induced diabetic and normal mice. The hot aqueous extracts of BN (250 and 500 mg/kg, i.p.) and Anemarrhena (170 and 340 mg/kg, i.p.) significantly promoted salivary flow in the diabetic animals, but suppressed it in the normal controls. An-S-2 and An-S-3 but not An-S-1 (10 mg/kg, i.p.), significantly promoted salivary flow in the diabetic animals. The potency order was An-S-3 ≫ An-S-2 ≫ extract. The hot aqueous extracts of BN and Anemarrhena increased the protein content of saliva in a dose-dependent manner. Combination of An-S-3 (0.1 mg/kg, i.p.) with CaCl₂ (2 and 4 mg/kg, i.p.) potentiated salivary flow compared with the respective effect of each on its own. These results demonstrated that 1) An-S-3 was mainly responsible for saliva secretion of the hot aqueous extract, and 2) the effect of An-S-3 was potentiated by combination with calcium, suggesting combined effects of Byakko-ka-ninjin-to containing *Anemarrhena asphodeloides* and gypsum fiber (calcium).

**Key words** saliva secretion; streptozocin-diabetic mice; timosaponin-AIII; proto-timosaponin-AIII; CaCl₂; Byakko-ka-ninjin-to

The rhizomes of *Anemarrhena asphodeloides* have been prescribed in Byakko-ka-ninjin-to (BN, Bai-Hu-Jia-Renshen-tang) in addition to the roots of Panax ginseng, gypsum fiber, roots of liquorice, and rice to treat patients with diabetes mellitus (Xiao-ke). BN is one of the Kampo Hozai (blended medicines) used in traditional Chinese medicine. We have reported that BN reduces the blood glucose level and promotes pilocarpine-induced saliva secretion in streptozocin (STZ)-induced diabetic mice. The anti-hyperglycemic effects are frequently accompanied by saliva secretory responses. The rhizomes of *Anemarrhena asphodeloides* and gypsum fiber (calcium) are important constituents in the overall effects of BN. We have reported anti-hyperglycemic effects of the hot aqueous extract and three saponins (pseudoprotosaponin-AIII (An-S-1), proto-timosaponin-AIII (An-S-2) and timosaponin-AIII (An-S-3)) from the rhizome of *Anemarrhena asphodeloides* in STZ-diabetic mice. However, the saliva secretory responses have not yet been investigated.

In the present study, we examined the effects of hot aqueous extracts of BN, the rhizome of *Anemarrhena asphodeloides*, and its associated anti-hyperglycemic compounds on pilocarpine-induced saliva secretion in STZ-diabetic mice. We examined also the potentiating interaction between the components and CaCl₂ to explain the combined effects of Anemarrhena and gypsum fiber (calcium) in BN.

**MATERIALS AND METHODS**

**Animals** Male ddY mice (4-week-old) weighting 18—23 g were purchased from Japan SLC (Shizuoka, Japan). The mice were injected intravenously (i.v.) with a bolus dose of 150 mg/kg STZ (Sigma, St. Louis, MO, U.S.A.), and were used 4—5 weeks (8-week-old, 28—35 g) later. The animals were maintained in an air-conditioned room with light from 7 a.m. to 7 p.m. The room temperature (23±1°C) and humidity (55±5%) were controlled automatically. The blood glucose levels in STZ-diabetic mice were more than 200 mg/dl after fasting for 13—14 h.

**Collection of Total Saliva** The hot aqueous extracts and compounds derived from rhizomes of *Anemarrhena asphodeloides* were dissolved in saline and injected intraperitoneally (i.p.) into the STZ-diabetic mice after fasting for 13—14 h. After 2 h, the mice were anesthetized with 50 mg/kg (i.p.) sodium pentobarbital (Nembutal, Abbott Lab., North Chicago, IL, U.S.A.) and placed on a heating pad which was maintained at 37°C. A polyethylene tube (i.d. 1.5 mm, length: 20 mm) was surgically inserted into the trachea to keep the airways open. A second stimulation was produced by administration of pilocarpine (1 mg/kg, i.v.) 10 min after the first pilocarpine stimulation (0.1 mg/kg, i.v.). The saliva secreted following stimulation was collected in micro-capillary tubes (Microcaps, 20 µl, Drummond, PA, U.S.A.) which were placed under the tongue, and the volumes was measured at one minute intervals. The total volume of saliva over 20 min was estimated. At the end of saliva collection, the submandibular, parotid and sublingual glands were isolated and weighed. Salivary flow was estimated as the volume of saliva per unit wet weight of total salivary glands per min. The protein concentration in saliva was determined by the Lowry method using bovine serum albumin as a standard.

**Determination of Blood Glucose Levels** Blood samples (20 µl) were obtained from the orbital venous plexus using glass capillaries before and 2, 4 and 6 h after injection. Blood glucose was measured by the glucose oxidase method using a glucose analyzer (Type 2, Beckman, CA, U.S.A.). The anti-hyperglycemic activity

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was evaluated as a percentage of the fall in blood glucose, 
\((b-a) \times 100/(b-85)\) where \(b\) and \(a\) are the blood glucose 
levels before and after injection of the extracts and 
compounds, respectively, and the value 85 is the mean 
blood glucose in the fasted state (14 h) for normal mice.

**Materials** BN is composed of *Anemarrhena asphodeloides*, 
ginseng root, liquorice root, rice and gypsum fiber 
in the ratio of 5:3:2:8:15. Processed ginseng (collected 
in the autumn in Jilin, China), liquorice (collected in 
the autumn in Chifeng, China), anemarrhena (collected in 
the autumn in Hebei, China), gypsum (collected in Shandong, 
China) and rice (collected in the autumn in Japan) were 
used. All crude drugs were purchased from Tochimoto 
Tenkaido. BN and each crude drug were decocted at 100 °C 
with 20 and 30 volumes of distilled water and reduced to 
half volume without a water-cooler. After the extracts 
were filtered through gauze, the filtrate was lyophilized and then 
stored in a desiccator at 4 °C. The yields (w/w) of 
lyophilized extract were 24% for ginseng, 26% for 
Anemarrhena, 30% for liquorice, 10% for rice and 3% for 
gypsum. Preparation of hot aqueous extracts and the 
isoaat of pseudoproto-timosaponin-AIII (An-S-1), 
proto-timosaponin-AIII (An-S-2) and timosaponin-AIII 
(An-S-3) from rhizomes of *Anemarrhena asphodeloides* 
have already been described.

Pilocarpine hydrochloride was obtained from Wako 
Pure Chemical Industries, Osaka, Japan.

**Statistical Analysis** Significant differences were ana-
lyzed using one-way ANOVA and determined by Scheffe’s 
multiple-comparison test. All data are expressed as 
means ± S.E.M.

**RESULTS**

**Promotion of Pilocarpine-Induced Saliva Secretion by 
Hot Aqueous Extracts of BN in Diabetic but not Normal 
Mice** When pilocarpine (1 mg/kg, i.v.) was injected into 
STZ-diabetic and normal mice, the salivary flow rate 
reached a peak within 1—2 min and then decreased 
gradually. After predindsay of hot aqueous 
extracts of BN (125—500 mg/kg, i.p.), the peak values and 
duration of saliva secretion were greater in STZ-diabetic 
mice than in the normal mice and controls (without BN) 
and this effect was dose-dependent (Fig. 1).

In STZ-diabetic mice, the hot aqueous extracts, at doses 
of 250 and 500 mg/kg, significantly promoted saliva 
secretion, but reduced it in normal mice (Fig. 2A). The 
protein content of the saliva was also increased significantly 
at the same doses in the STZ-diabetic mice, but

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**Fig. 1.** Time-Dependent Potentiating Effects of Hot Aqueous Extracts 
from Byakkko-ka-ninjin-to (BN) on Pilocarpine-Induced Saliva Secretion 
In STZ-Diabetic Mice (Upper), and Inhibitory Effects in Normal Mice 
(Lower)

Saliva was collected at every minute intervals over a 20-min period following 
the second stimulation with pilocarpine (1 mg/kg, i.v.). The rate of salivary flow 
was estimated. Open circles represent saline controls. Closed circles represent mice 
treated with hot aqueous extract (125—500 mg/kg, i.p.). The values are means ± 
S.E.M. of 6—14 mice.

**Fig. 2.** Dose-Dependent Potentiating Effects on Pilocarpine-Induced 
Saliva Secretion by Hot Aqueous Extract of BN in STZ-Diabetic Mice 
and Inhibitory Effects in Normal Mice

A: salivary flow and B: protein content of saliva. Saliva was collected over a 
20-min period after the second stimulation with pilocarpine (1 mg/kg, i.v.). Closed 
and open circles represent effects, with and without BN (125—500 mg/kg, i.p.), on 
pilocarpine-induced saliva secretion in normal and STZ-diabetic mice, respectively. 
The values are means ± S.E.M. of 8—12 mice, *p < 0.05 and **p < 0.01; 
significantly different from normal mice by one-way ANOVA and then Scheffe’s 
multiple-comparison test.
not in normal mice (Fig. 2B). The extract alone stimulated neither the basal salivary flow nor increased the basal protein content of the saliva.

Promotion of Effects on the Pilocarpine-Induced Saliva Secretion by Hot Aqueous Extract of Anemarrhena and Ginseng Included in BN The effects of hot aqueous extract from Anemarrhena (170 mg/kg), ginseng (90 mg/kg), liquorice (80 mg/kg), rice (100 mg/kg) and gypsum (60 mg/kg) on pilocarpine-induced saliva secretion were examined. The crude drugs were injected (i.p.) into STZ-diabetic mice at the dose ratio used BN. Both Anemarrhena and ginseng significantly promoted salivary flow compared with the value for the controls (Fig. 3A). The protein content of the saliva was increased significantly by Anemarrhena and rice (Fig. 3B).

Potentiating Effects of a Hot Aqueous Extract from Anemarrhena and Its Component Compounds, An-S-2 and An-S-3, on Pilocarpine-Induced Saliva Secretion in Diabetic Mice The dose-dependent effects of an extract from Anemarrhena on salivary flow and the protein content of the pilocarpine-induced saliva secretion in diabetic mice were compared with those in normal mice. In STZ-diabetic mice, the extract of Anemarrhena potentiated significantly the salivary flow and increased the protein content of the saliva at doses of 170 and 340 mg/kg, but inhibited salivary flow at a dose of 340 mg/kg in normal mice (Fig. 4).

The potentiating effects of An-S-1, An-S-2 and An-S-3 at a dose of 10 mg/kg (i.p.) on pilocarpine-induced saliva secretion were examined in STZ-diabetic mice. Both An-S-2 and An-S-3 significantly promoted salivary flow compared with controls, but did not change the protein content of the saliva (Fig. 5). An-S-1 had no effect on saliva secretion. These compounds alone at 10 mg/kg (i.p.) stimulated neither the basal salivary flow nor increased the basal protein content of saliva.

The potentiating effect on salivary flow was ranked as An-S-3 ≫ An-S-2 ≫ extract (Table 1).

Dose-Dependent Effect of An-S-3 and the Potentiating Effects of An-S-3 and CaCl₂ on Pilocarpine-Induced Saliva Secretion The dose-dependent effects of An-S-3 on salivary flow and the protein content of pilocarpine-induced saliva secretion were examined at 0.1, 1 and 10 mg/kg (i.p.). An-S-3 significantly promoted salivary flow at a dose of 1 and 10 mg/kg in STZ-diabetic mice.
Fig. 5. Potentiating Effects of An-S-2 and An-S-3 on Pilocarpine-Induced Salivary Flow (A) and Protein Content (B) in the Saliva of STZ-Diabetic Mice

Saliva was collected as shown in Fig. 2. Open columns represent saline controls. Hatched columns represent mice injected with An-S-1, An-S-2 and An-S-3 (10 mg/kg, i.p.). The values are means ± S.E.M. of 8–15 mice. **p<0.01; significantly different from saline controls by one-way ANOVA and then Scheffe’s multiple-comparison test.

(Fig. 6A).

The potentiating effects of An-S-3 (0.1–10 mg/kg, i.p.) with respect to CaCl₂ (2 mg/kg, i.p.) on pilocarpine-induced saliva secretion were examined. The combination of CaCl₂ (2 mg/kg) with An-S-3 (0.1 and 1 mg/kg) significantly promoted salivary flow (Fig. 6A), but did not increase the protein content of the saliva (data not shown), compared with the values in mice injected with An-S-3 alone. The potentiating effects of CaCl₂ (0.5–4 mg/kg, i.p.) with respect to An-S-3 (0.1 mg/kg, i.p.) on pilocarpine-induced saliva secretion were examined. An-S-3 at 0.1 mg/kg and CaCl₂ at 0.5 to 4 mg/kg did not promote saliva secretion on their own. However, combination of An-S-3 (0.1 mg/kg) with CaCl₂ (2 and 4 mg/kg) significantly promoted salivary flow (Fig. 6B), but did not increase the protein content of the saliva (data not shown), compared with the values in mice injected with CaCl₂ alone.

Comparison between the Potentiating Effects on Pilocarpine-Induced Saliva Secretion and Anti-hyperglycemic Effects of Hot Aqueous Extracts of BN, Anemarrhena and Its Component Compounds An-S-1, An-S-2 and

Fig. 6. The Dose-Dependent Potentiation of An-S-3 and CaCl₂ on Pilocarpine-Induced Saliva Secretion in STZ-Diabetic Mice

Saliva was collected as shown in Fig. 2. The dose-dependent potentiation of An-S-3 combined with CaCl₂ (2 mg/kg, i.p.) (A) and of CaCl₂ combined with An-S-3 (0.1 mg/kg, i.p.) on pilocarpine-induced saliva secretion in STZ-diabetic mice. Closed and open circles represent the combined and individual effects, respectively. The values are means ± S.E.M. of 8–12 mice. *p<0.05 and **p<0.01; significantly different vs. individual effects by one-way ANOVA and then Scheffe’s multiple-comparison test.

An-S-3 We observed significant potentiating effects by BN (500 mg/kg, i.p.) on pilocarpine-induced saliva secretion 2 h after its injection (Fig. 7). At the same doses, BN showed a significant anti-hyperglycemic effect 4 and 6 h after its injection. The effects increased with time leading to a 50% fall in blood glucose 6 h after injection. After promoting saliva secretion there was an interval of 2 h before the onset of the anti-hyperglycemic effects.

The required doses for a 20% increase (ED₂₀) in pilocarpine-induced saliva secretion and a 50% fall (ED₅₀) in blood glucose were compared for the hot aqueous extracts of BN and Anemarrhena, and its component compounds An-S-1, An-S-2 and An-S-3 in STZ-diabetic mice (Table 1). As far as saliva secretion was concerned, An-S-2 and An-S-3 were more potent than the hot aqueous extracts of BN and Anemarrhena. In particular the effects of An-S-3 on salivary flow and protein content were the most potent of the compounds tested. The anti-hyperglycemic effects of the compounds did not reflect the saliva secretory responses. An-S-1 exhibited the most potent anti-hyperglycemic effects.
DISCUSSION

BN used clinically to treat diabetes mellitus is a traditional medicine. We have reported that hot aqueous extracts from each crude component of BN exhibit anti-hyperglycemic activity in diabetic mice.\(^2,3\) The rhizome of *Anemarrhena asphodeloides* is the main crude drug in BN.\(^4\) The component compounds are tinosaponin-AI, -AII, -AIII, -AIV, -BI and -BII.\(^9-11\) As non-saponin compounds, four glycosans (anemaranas A, B, C, and D) have been isolated from *Anemarrhena asphodeloides* rhizomes, and are hypoglycemic in normal and anti-hyperglycemic in alloxan-diabetic mice.\(^12\) In the present study, we showed that the hot aqueous extracts of BN and Anemarrhena, and its component compounds An-S-2 and An-S-3 with anti-hyperglycemic effects,\(^3\) promote pilocarpine-induced saliva secretion in STZ-diabetic mice. Both extracts of BN and Anemarrhena were effective only in diabetic animals but had a rather inhibitory effect in normal animals although the reason is unknown.

The yields of the hot aqueous extracts of Anemarrhena, An-S-1, An-S-2 and An-S-3 were 39%, 0.04%, 0.23% and 0.17% from the crude drug, respectively.\(^5\) An-S-1 at 10 mg/kg (i.p.) had no effect on saliva secretion, but was the most potent anti-hyperglycemic among the three compounds. An-S-3, for saliva flow, was 175-fold more potent than the hot aqueous extracts. Therefore, An-S-1 and An-S-3 may play key roles in the anti-hyperglycemic effect and the saliva secretion, respectively, produced by hot aqueous extracts of Anemarrhena in STZ-diabetic mice.

The volume of saliva flow is dependent on the intracellular Ca\(^{2+}\) concentration in the acinar cells of the salivary gland.\(^13\) Our data indicate that the administration of CaCl\(_2\) potentiates pilocarpine-induced saliva secretion, not on its own but in combination with An-S-3. These results suggest that the rhizomes of *Anemarrhena asphodeloides* and gypsium (calcium) may be responsible for the potentiation effects on saliva secretion in STZ-diabetic mice.

The present data indicate that BN significantly promotes saliva secretion after 2 h and anti-hyperglycemic effects after 6 h, following its injection into diabetic mice. The accelerated saliva secretion and subsequent anti-hyperglycemic effect may be due to a time-lag in the effects of BN. A strong correlation has been observed between anti-hyperglycemic effects and pilocarpine-induced saliva secretion produced by extracts of mulberry leaves and its component compound, GAL-DNJ.\(^9\) The peptide P-C, consisting of 44 amino acid residues from the human parotid gland, shows anti-hyperglycemic effects in diabetic mice.\(^14,15\) These results suggest that some secretory substances in saliva may cause anti-hyperglycemic effects.

In conclusion, the hot aqueous extract of BN and Anemarrhena, and component, An-S-3, significantly promoted pilocarpine-induced salivary flow in STZ-diabetic mice. An-S-3 mainly contributed to the saliva secretion.
secretion promoted by the hot aqueous extract, which was potentiated by combination with calcium, suggesting a combined effect with BN containing Anemarrhena asphodeloides and gypsum fiber (calcium).

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REFERENCES

13) Foskett J. K., Melvin J. E., Science, 244, 1582—1585 (1989).