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Combined effect of total alkaloids from *Feculae Bombycis* and natural flavonoids on diabetes

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Abstract

Both total alkaloids from *Feculae Bombycis* (TAFB) and natural flavonoids can inhibit α-glucosidase activity to depress the glucose level in blood. To investigate the cooperative effect of TAFB and flavonoids on blood glucose, we have studied their combined function compared with individual ingredients on enzymology, in-vitro and in-vivo. In the enzymological assay, the combination of TAFB and flavonoids showed more effective inhibition, compared with either TAFB or flavonoids alone, to α-glucosidase activity. In the everted intestine model in-vitro, the combined inhibition of starch hydrolysation and glucose transference to blood was much stronger than with separate components. In short-term studies with normal and experimentally-induced diabetic mice in-vivo, the combination of TAFB and flavonoids also had a stronger suppressive effect on the postprandial elevation in blood glucose after oral administration. In long-term treatment to diabetic mice in-vivo, the compound prescription could depress not only the fasting blood glucose, but also the fasting blood total cholesterol. These results demonstrated that TAFB and flavonoids could inhibit α-glucosidase activity cooperatively, which would successfully depress blood glucose level in the therapy of diabetes.

Introduction

α-Glucosidase inhibitors (AGIs; acarbose, miglitol, voglibose) are widely used in the treatment of patients with type 2 diabetes. AGIs delay the absorption of carbohydrates from the small intestine and thus have a lowering effect on postprandial blood glucose and insulin levels (Laar et al 2005).

Feculae Bombycis (silkworm feces) is a Chinese traditional and herbal drug. We have investigated total alkaloids from *Feculae Bombycis* (TAFB), which has a main component of 1-deoxynojirimycin (DNJ) (Geng et al 2005). DNJ is a typical natural alkaloid (Asano et al 1994a, b, 2001), with a promising strong inhibitory action on α-glucosidase activity. DNJ and similar N-containing sugars, present in mulberry and silkworm resources, are well-known potent AGIs (Miyahara et al 2004).

Flavonoids, which are widely distributed in the plant kingdom and present in considerable quantities in common food products, spices and beverages, have been used since ancient times by physicians and laymen to treat a great variety of human diseases, such as diabetes, coronary heart disease and cancers (Havsteen 1983). As AGIs, however, there have been only a few reports on flavonoids in recent years (Kim et al 2000; Kawabata et al 2003; Gao et al 2004; Tanaka et al 2004).

Polyphenols from berries have an obvious effect on digestive enzymes (McDougall & Stewart 2005), catechin is a potent AGI (Hakamata et al 2006) and quercetin is reported to alleviate the activity of intestinal and renal disaccharidases in streptozotocin-induced diabetic rats (Ramachandra et al 2005). These three flavonoids are all potent AGIs.

It is important to use compound prescriptions besides single ones in Chinese traditional and herbal drugs. However, there have been no reports on the research of compound AGIs. In this study we investigated the cooperation of two kinds of AGIs, TAFB and flavonoids, which provided a new idea in the therapy of diabetes.

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Materials and Methods

Drugs and reagents

TAFB was prepared in our laboratory (containing alkaloid 82.6%) (Geng et al 2005); catechin and quercetin were purchased from Institute For Drug Control (Tianjin, China); alloxan was from Sigma-Aldrich (St Louis, MO). α-Glucosidase (EC 3.2.1.20) was obtained from Wako Pure Chemical Industries, Ltd (Japan); acarbose (Glucobay) was from Bayer Healthcare Co., Ltd (Germany); Glucose Test kit (GOD-PAP) and Total Cholesterol Test kit (CHOD-PAP) were from Zhong Sheng Bio-technology and Science Co., Ltd (Beijing, China).

Animals

This study was carried out using 72 Kunming mice (18~22 g, 6 weeks old) and Wistar rats (120~140 g, 4 weeks old) from the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). Rats were maintained in a clean room at a temperature between 23~26°C and a relative humidity of 50~60% with a 12-h light–dark cycle. Both sexes were used. They were housed in separate cages with food and water freely available. The experimental protocol was approved by the Animal Ethics Committee of Nankai University, in accordance with Principles of Laboratory Animal Care and Use in Research (Ministry of Health, Beijing, China).

HPLC for DNJ determination

The method was reported by Kim et al (2003). DNJ was derivatized with 9-fluorenylmethyl chloroformate (FMOC-Cl), and analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a fluorescence detector.

Extraction of tea polyphenol (TP) from green tea

Green tea (16 g) was extracted (20 min) with 70% aqueous methanol (v/v) under stirring. The solvent was evaporated in-vacuo and the residue was twice extracted with 300 mL of ethyl acetate. The aqueous phase was discarded. The pooled organic layer was evaporated in-vacuo and freeze-dried. The obtained solid was used in the subsequent experiments (Degenhardt et al 2000).

Inhibition of the activity of α-glucosidase

The assay mixture consisted of 100 μL 10 mg mL^{-1} maltose as substrate and 200 μL 0.1 mg mL^{-1} different α-glucosidase inhibitor. The mixture was pre-incubated for 5 min at 37°C, and the reaction was initiated by adding 100 μL α-glucosidase (6 IU mL^{-1} α-glucosidase, in 20 mM NaH_2PO_4-Na_2HPO_4, pH 7.0), containing 150 mM NaCl and 1% BSA, into the reaction mixture. This mixture was incubated for 20 min at 37°C, and the reaction was terminated by heating for 2~3 min in a boiling water bath. The glucose released in the reaction mixture was determined by a Glucose Test kit based on the glucose oxidase method. The reaction mixture (10 μL) and Glucose Test kit (150 μL) were mixed and incubated for 15 min at 37°C. The absorbance of the mixture was measured at 490 nm by an ELISA reader. The value indicates the activity of α-glucosidase in different conditions.

The rat everted intestinal sac system in-vitro

The experiment was performed according to the method of Vogel & Vogel (2001) with a minor modification. Under pentobarbital anaesthesia, the intestine was excised from a 7-week-old rat after an overnight fast. Then the inside of the intestine was rinsed twice with ice-cold Krebs-Henseleit solution. After being cut into several segments about 4 cm long, the intestine was then everted by inserting a piece of glass tubing and tying with cotton thread at one end. The other end was tied off with thread so as to form a sac, with the serosal side facing inwards. This sac was filled with 200 μL of the Krebs-Henseleit solution. Each sac preparation was suspended in a tube with 4 mL of the Krebs-Henseleit solution containing 1% starch and the required concentration of each drug and immersed in a water bath to incubate for 60 min at 37°C. The reaction was terminated by adding 10 μL 1 M HCl into the tube. At the end of the experiment, the solutions from the serosal side (for absorption and transference to blood) and the mucosal side (for reaction in intestine) were both collected to measure the concentration of glucose by the Glucose Test kit. Comparing every group's glucose to the control, the value E/E_0 indicated starch hydrolysation or glucose transference to blood in different conditions. Data were analysed using a one-way analysis of variance test. Individual differences between the compounds were then evaluated using Tukey's test.

Effect on postprandial elevation in blood glucose of mice

The mice were used for the oral administration experiment after food deprivation for 16 h. The different samples and starch (5 g kg^{-1}) were simultaneously orally administered to the mice. Blood samples (20 μL) were collected before and 30, 60 and 120 min after administration from the tail vein of each mouse. The plasma was separated from the collected blood, and the concentration of glucose was measured by the Glucose Test kit. Data were analysed using a two-way repeated analysis of variance test. Individual differences between the compounds were then evaluated using Tukey's test.

Models of experimental-diabetic mice

The diabetic mice were set up by receiving alloxan (200 mg kg^{-1}, i.p.) after food deprivation for 16 h. The difference in average fasting blood glucose level between each group was less than 0.5 mM.

Long-term therapy in diabetic mice

The samples were orally administered after 5 h of food deprivation to the experimental-diabetic mice once a day for 3

weeks. Food and water were freely available during other periods. At the end of the experiment, the fasting blood glucose and total cholesterol of each mouse were measured by the Glucose Test kit and the Total Cholesterol Test kit after food deprivation for 16 h. Data were analysed using a one-way analysis of variance test. Individual differences between the compounds were then evaluated using Tukey's test.

Results

Determination of DNJ in TAFB

The HPLC chromatogram of DNJ reference substance (Figure 1A) was composed of DNJ-FMOC, FMOC-derivatized glycine (Gly-FMOC) and hydrolysed FMOC-Cl (FMOC-OH). The chromatogram of TAFB (Figure 1B) showed that the main alkaloid in the extract was DNJ (56.7%, w/w).

Cooperative inhibition by TAFB and flavonoids of α-glucosidase

The inhibition by TAFB and flavonoids of the activity of α-glucosidase was first measured on the level of enzymology. TAFB and flavonoids, including catechin, quercetin and TP, could separately inhibit α-glucosidase activity compared with the control (Figure 2), and the capacity of TAFB was stronger than flavonoids. Meanwhile, the inhibition of the combination of TAFB and flavonoids was stronger than the individual compounds, which indicated that TAFB and flavonoids could inhibit α-glucosidase cooperatively.

Cooperative inhibition by TAFB and flavonoids of starch hydrolysation and glucose transference using the everted intestine model in-vitro

The cooperative inhibition by TAFB and flavonoids of α-glucosidase was further tested in-vitro using the rat everted intestinal sac system. The samples each showed significant inhibition of starch hydrolysation ($P < 0.01$) and glucose transference to blood ($P < 0.01$, $P < 0.05$) compared with the control (Table 1). Furthermore, the TAFB + quercetin group and the TAFB + TP group both showed significantly ($P < 0.01$ and $P < 0.05$, respectively) stronger inhibition of starch hydrolysation compared with the TAFB group. Meanwhile, the TAFB + catechin group showed significantly ($P < 0.05$) stronger inhibition of glucose transference to blood compared with the TAFB group. These results indicated that the combination of TAFB and flavonoids (quercetin in particular) had obvious synergies. The combined inhibition of starch hydrolysation and glucose transference to blood was much more effective than the inhibition by the individual ingredients.

Suppressive effect of TAFB and flavonoids on the postprandial elevation in blood glucose of normal mice

The cooperative inhibition by TAFB and flavonoids of α-glucosidase in-vivo was investigated on the basis of the in-vitro results. After oral starch administration, the blood glucose level in the control group showed a maximum value after 30 min (Table 2). TAFB and flavonoids (catechin, quercetin), however, significantly ($P < 0.01$, $P < 0.05$) depressed the blood glucose level at the 30-min point after starch loading compared with the control. The TAFB + catechin and TAFB + catechin + quercetin groups even caused significant ($P < 0.01$ and $P < 0.05$, respectively) depression at the 60-min point. Furthermore, the TAFB + catechin and TAFB + catechin + quercetin groups also displayed a significantly ($P < 0.05$) stronger suppressive effect compared with the TAFB group. These results suggested that TAFB and flavonoids could not only depress the postprandial blood glucose level in normal mice, but also react cooperatively when combined.

Suppressive effect of TAFB and flavonoids on the postprandial elevation in blood glucose of experimental-diabetic mice

The further synergistic inhibition by TAFB and flavonoids of α-glucosidase was investigated in experimental-diabetic

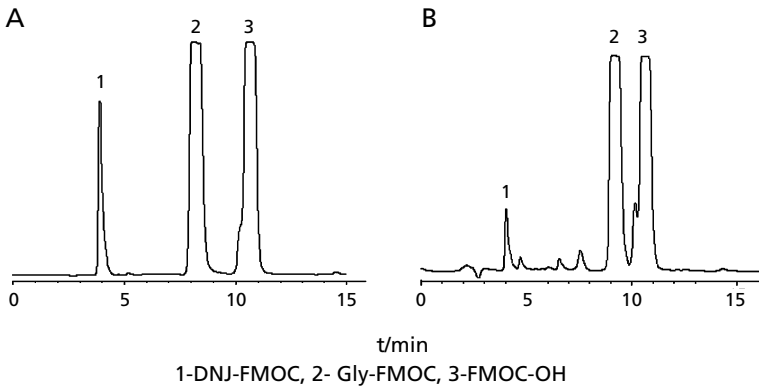

Figure 1 HPLC chromatograms of DNJ reference substance (A) and TAFB (B).

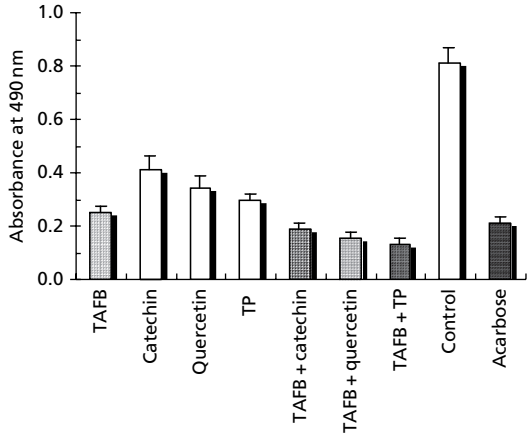

Figure 2 The cooperative inhibition by TAFB and flavonoids of α-glucosidase. Results are mean ± s.d., n = 5.

Table 1 Inhibition by TAFB and flavonoids of starch hydrolysation and glucose transference in the rat everted intestine experiment

Group	Dose (mg mL^{-1})	Starch hydrolysation E/E_0 (%)	Glucose transference E/E_0 (%)
Control	—	100.0	100.0
Acarbose	0.1	26.8 ± 9.2**	42.1 ± 20.0**
TAFB	0.1	21.7 ± 10.9**	32.0 ± 14.2**
Catechin	1.0	64.5 ± 10.3**	75.9 ± 20.4*
Quercetin	1.0	46.6 ± 8.6**	74.2 ± 12.1**
TP	1.0	19.2 ± 7.3**	64.1 ± 16.3**
TAFB + catechin	0.1 + 1.0	14.2 ± 10.2**	18.8 ± 6.8**$^{+}$
TAFB + quercetin	0.1 + 1.0	4.0 ± 2.7**$^{++}$	20.8 ± 9.2**
TAFB + TP	0.1 + 1.0	9.2 ± 4.8**$^{+}$	40.6 ± 10.0**

Results are mean ± s.d., n = 5. *$P < 0.05$ and **$P < 0.01$ compared with control group; $^{+}P < 0.05$ and $^{++}P < 0.01$ compared with TAFB group.

mice. As the results in Table 3 show, the mice in the control group, which had received alloxan previously, showed significantly ($P < 0.01$) higher blood glucose levels at each time point compared with the normal group. After oral starch administration, the blood glucose level in the control group showed maximum value during the 30~60-min period. However, the TAFB and TAFB + quercetin groups had a significantly ($P < 0.01$) depressed maximum level of blood glucose compared with the control group. Furthermore, the TAFB + quercetin group seemed to show a stronger suppressive effect than the TAFB group, though there was no significant difference in statistical analysis. These results indicated that TAFB could not only depress the postprandial elevation in blood glucose of experimental-diabetic mice, but also reveal cooperation when combined with flavonoids (quercetin).

Long-term studies of the compound prescription (TAFB–quercetin, 1:2) on the fasting blood glucose and total cholesterol in diabetic mice

To investigate the inhibition by compound prescription (TAFB–quercetin, 1:2) of α-glucosidase in-vivo in the long term, we observed the effect on diabetic mice after 3 weeks' therapy. The fasting blood glucose levels in the 30 mg kg^{-1} and 90 mg kg^{-1} compound prescription groups were both significantly ($P < 0.01$ and $P < 0.05$, respectively) lower compared with the control group (Table 4). Meanwhile, the depression in the 30 mg kg^{-1} compound prescription group was significantly ($P < 0.05$) stronger than in the acarbose group at the same dose. However, the fasting blood glucose in the 90 mg kg^{-1} compound prescription group was unexpectedly higher than in the 30 mg kg^{-1} group. Although the fasting blood total cholesterol in experimental-diabetic mice that were set up by receiving alloxan would not rise obviously, the 90 mg kg^{-1} compound prescription group showed a significantly ($P < 0.05$) lower level compared with the control group. These results suggested that the compound prescription of TAFB and quercetin combined could not only depress the fasting blood glucose in experimental-diabetic mice after 3 weeks' therapy, but also depress the fasting blood total cholesterol.

Discussion

The main alkaloid in TAFB is DNJ, the famous natural AGI (Asano et al 1994a, b, 2001; Kimura & Chen 1995). TAFB,

Table 2 Suppressive effect of TAFB and flavonoids on the postprandial elevation in blood glucose of normal mice

Group	Dose (mg mL^{-1})	Blood glucose (mM)			
		0 min	30 min	60 min	120 min
Control	—	4.52 ± 0.39	10.41 ± 1.17	7.53 ± 1.34	5.12 ± 0.89
Acarbose	4.0	3.99 ± 0.53	7.31 ± 1.38**	7.48 ± 1.14	5.60 ± 0.95
Catechin	8.0	4.49 ± 0.93	7.93 ± 1.43**	8.34 ± 2.37	5.61 ± 0.72
Quercetin	8.0	4.28 ± 0.62	8.15 ± 1.86*	8.09 ± 1.63	6.11 ± 1.06
TAFB	4.0	4.08 ± 0.25	7.46 ± 1.35**	6.01 ± 1.90	5.01 ± 1.15
TAFB + catechin	4.0 + 8.0	4.54 ± 0.69	6.05 ± 0.87**$^{+}$	5.38 ± 1.03**	5.24 ± 0.64
TAFB + quercetin	4.0 + 8.0	3.81 ± 0.47	6.49 ± 1.18**	6.58 ± 0.79	4.89 ± 0.95
TAFB + catechin + quercetin	4.0 + 8.0 + 8.0	4.48 ± 0.42	5.66 ± 1.65**$^{+}$	5.82 ± 1.59*	5.73 ± 1.27

Results are mean ± s.d., n = 7. *$P < 0.05$ and **$P < 0.01$ compared with control group; $^{+}P < 0.05$ compared with TAFB group.

Table 3 Suppressive effect of TAFB and flavonoids on the postprandial elevation in blood glucose of experimental-diabetic mice

Group	Dose (mg mL^{-1})	Blood glucose (mM)			
		0 min	30 min	60 min	120 min
Normal	—	4.52 ± 0.39	10.41 ± 1.17	7.53 ± 1.34	5.12 ± 0.89
Control	—	15.01 ± 1.59^{++}	21.27 ± 1.94^{++}	20.11 ± 2.88^{++}	15.63 ± 1.17^{++}
Acarbose	4.0	13.75 ± 0.64	16.46 ± 2.16**	15.93 ± 1.63**	12.41 ± 0.79
TAFB	4.0	14.37 ± 1.51	17.29 ± 2.11**	17.27 ± 2.74	12.55 ± 2.33
TAFB + quercetin	4.0 + 8.0	15.20 ± 0.83	15.78 ± 1.66**	16.24 ± 1.78**	15.82 ± 0.92

Results are mean ± s.d., n = 7. $^{++}P < 0.01$ compared with normal group; $^{**}P < 0.01$ compared with control group.

Table 4 Effect of the compound prescription on the fasting blood glucose and total cholesterol in diabetic mice

Group	Dose (mg kg^{-1})	n	Glucose (mM)	Cholesterol (mM)
Normal	—	11	6.50 ± 1.26	3.90 ± 1.33
Control	—	9	22.38 ± 8.76^{++}	3.95 ± 0.85
Acarbose	30	11	16.74 ± 7.59	3.76 ± 0.59
Compound prescription (TAFB–quercetin, 1:2)	10	10	22.55 ± 4.98	3.50 ± 0.55
	30	10	11.01 ± 3.82**$^{\&}$	3.67 ± 1.18
	90	9	15.52 ± 7.24*	3.09 ± 0.50*

Results are mean ± s.d., $^{++}P < 0.01$ compared with normal group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group; $^{\&}P < 0.05$ compared with acarbose group.

therefore, can inhibit α-glucosidase efficiently. Four other alkaloids, fagomine, 3-*epi*-fagomine, 1,4-dideoxy-1,4-imino-D-arabinitol and 1,4-dideoxy-1,4-imino-(2-*O*-β-D-glucopyranosyl)-D-arabinitol, were reported in the silkworm's body by Asano et al (2001). Accordingly, they should exist in TAFB, the silkworm's feces. Fagomine, among them, contributes little to the inhibition of α-glucosidase. However, fagomine is able to potentiate glucose-induced insulin secretion (Asano et al 2001). This effect would make TAFB depress glucose levels more effectively in long-term studies in-vivo.

On the other hand, because of the restriction of materials, we only found three kinds of flavonoids mentioned in this study (catechin, quercetin and TP) that were able to inhibit α-glucosidase obviously. Regretfully, lots of other kinds of flavonoids in our study, such as rutin and hesperidin, did not show distinct inhibition of α-glucosidase. Hence, it is necessary to extend the range studied to find stronger AGIs in the flavonoid family. This will offer more choices in the combination with TAFB.

Among the three kinds of flavonoids, TP showed the strongest inhibition of α-glucosidase (Figure 2) and starch hydrolysation (19.2 ± 7.3%, Table 1). When combing with TAFB (Table 1), however, TP inhibited starch hydrolysation (9.2 ± 4.8%) and glucose transference (40.6 ± 10.0%) less effectively compared with quercetin (4.0 ± 2.7% and 20.8 ± 9.2, respectively). Meanwhile, TP could obviously accelerate glucose absorption, therefore the inhibition of glucose transference (Table 1) by TP + TAFB (40.6 ± 10.0%) was even weaker than individual TAFB (32.0 ± 14.2%). For these reasons, we did not investigate TP in-vivo, and did not choose it in the final combination.

Quercetin is another famous natural compound against diabetes. Most research has focused on its alleviation of antioxidative stress in diabetes (Lean et al 1999; Sanders et al 2001; Coldiron et al 2002; Vessal et al 2003; Mahesh & Menon 2004). However, there are few reports on its inhibition of α-glucosidase, except for one by Ramachandra et al (2005). In our study, quercetin was regarded as an AGI. Quercetin and quercetin + TAFB both showed stronger inhibition of α-glucosidase (Figure 1) and starch hydrolysation (Table 1, 46.6 ± 8.6% and 4.0 ± 2.7%, respectively) compared with catechin (64.5 ± 10.3% and 14.2 ± 10.2%, respectively). Furthermore, the combination of quercetin and TAFB displayed notable suppressive effects on high blood glucose level in short- and long-term studies in-vivo (Table 2, 3, 4). All these results demonstrated the cooperation of TAFB and quercetin in the inhibition of α-glucosidase activity. Moreover, the presence of quercetin could also contribute additional bioactivity against diabetes, such as inhibition of haemoglobin glycosylation (Asgary et al 1999), protection against beta-cell damage (Coskun et al 2005), attenuation of diabetic nephropathy (Anjaneyulu & Chopra 2004), partial reversal of impaired cardiac function (Krishna et al 2005), and so on. The multi-bioactivity could further enhance the suppressive effects on high blood glucose level.

In this study, we investigated the combination of different kind of natural AGIs, confirmed the cooperation between TAFB and flavonoids and developed modern compound Chinese traditional and herbal drugs against diabetes. The first member, TAFB, was a kind of polyhydric alkaloid from mulberry and silkworm resources. Its inhibition of starch and oligosaccharide hydrolysation was quite effective. The other member was a kind of flavonoid, which had a multi-bioactivity against diabetes as well as being an AGI. Because of the entirely different chemical structures of the two kinds of AGIs, their inhibitory mechanisms of α-glucosidase may be distinct. The difference includes the binding site to α-glucosidase, the interacting amino acid residues, the induced enzymatic molecular allostery, and so on. Once the two kinds of AGIs bind to the α-glucosidase molecule simultaneously, synergistic inhibition may happen. The hypothesis should be confirmed

by compelling data. The relative research, such as kinetic and structural analysis of enzyme–inhibitor complex, is now in progress.

Conclusions

Total alkaloids from *Feculae Bombycis* (TAFB) and flavonoids can inhibit the activity of α-glucosidase cooperatively when combined at the level of enzymology, in-vitro and in-vivo. The compound prescription of combined TAFB and quercetin can depress blood glucose level notably when used in the therapy of diabetes.

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