

Anti-Diabetic Effects of Pumpkin and Its Components, Trigonelline and Nicotinic Acid, on Goto-Kakizaki Rats

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The effects of a pumpkin paste concentrate and its components on oral glucose tolerance and serum lipid levels were determined in non-obese type 2 diabetic Goto-Kakizaki (GK) rats. In the oral glucose tolerance test, the pumpkin paste concentrate-fed group maintained a lower glucose level than the control group between 15 and 60 min. The compounds considered to be effective in improving glucose tolerance and contained in the methanol extract of the pumpkin in relatively abundant amounts were isolated and identified as trigonelline (TRG) and nicotinic acid (NA).

Feeding a diet containing TRG and NA respectively improved and tended to improve glucose tolerance. The insulin level increased after 15 min in the TRG-fed GK rats and then gradually decreased over the next 120 min. In contrast, a gradual increase was seen in the insulin level over 120 min in the control GK rats not fed with TRG, suggesting that TRG could improve the insulin resistance. The serum and liver triglyceride (TG) levels in the TRG- and NA-fed GK rats were lower than those in the control GK rats. Lower activity of liver fatty acid synthase (FAS), and higher activity of liver carnitine palmitoyl transferase (CPT) and glucokinase (GLK) in the TRG- and NA-fed GK rats than in the control GK rats were observed. This suggests that the regulation of these enzyme activities by TRG and NA was closely related to the suppression of both TG accumulation and the progression of diabetes.

Key words: pumpkin; trigonelline; nicotinic acid; Goto-Kakizaki rats

Lifestyle-related diseases such as dyslipidemia, arteriosclerosis, diabetes mellitus and hypertension, which are mainly caused by eating habits and lifestyle choices, are a socially important problem. This is because they are widespread and prevalent diseases in industrialized countries, and concern exists that they are closely linked to an increase in mortality. These diseases are contributory causes in about half of the deaths in industrialized countries. The prevalence of type 2 diabetes mellitus (T2DM) is predicted to increase dramatically in the next few years. T2DM is a multifaceted disorder character-

ized by hyperglycemia with insulin resistance and impaired insulin secretion, resulting in a decreased peripheral glucose uptake and increased blood glucose level. Since T2DM causes serious complications of the nervous system,¹⁾ kidney,²⁾ and eye,³⁾ its prevention and treatment are urgent priorities.

While the pumpkin is a vegetable which is widely consumed, there are few reports dealing with its functionality as a foodstuff. Quanhong *et al.* have reported that the protein-bound polysaccharide in pumpkin decreased the serum insulin level in type I diabetes.⁴⁾ It has also been reported that pumpkin-seed oil was able to suppress an increase in blood pressure,⁵⁾ and to decrease the blood lipid level.⁶⁾ However, the other compounds contained in pumpkin and their expected antidiabetic effects, especially those with low molecular weight, have not been investigated in relation with their chemical structures.

A pumpkin paste concentrate was prepared by decocting both the water-soluble and fine particle parts of a pumpkin homogenate for this being considered to contain low-molecular-weight compounds which are effective mitigating diabetes. The anti-diabetic effects of the pumpkin paste concentrate and its active principles were determined in the genetically modified T2DM GK rat model by measuring such relevant markers of diabetes as glucose tolerance, insulin resistance and the hemoglobin A1c, insulin, fasting glucose and adipocytokine levels, and further enzyme activities concerned with glucose and lipid metabolism.

Materials and Methods

Preparation of the pumpkin paste concentrate. The edible portion of raw pumpkin (600 g) homogenized with 2 volumes of water by a Mascolloider (Masuko Sangyo Co., Saitama, Japan) was centrifuged with a screw decantor to remove the large particles. The supernatant was boiled at 99 °C for 10 min and then concentrated at 60–70 °C under reduced pressure to obtain a yield of 100 g (36% Brix value). The concentrate passed through a filter with 150- μ m pores (the pumpkin paste concentrate) was used as pumpkin in animal experiments after it had been freeze-dried. The obtained pumpkin paste concentrate was composed of 33 g of water, 12 g of protein, 0.6 g of lipid, 50.2 g of carbohydrate, and 4.2 g of ash and other material. The sample used for

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Abbreviations: TRG, trigonelline; NA, nicotinic acid; TG, triglyceride; FAS, fatty acid synthase; CPT, carnitine palmitoyl transferase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GLK, glucokinase; G6Pase, glucose-6-phosphatase

the animal experiments was prepared by lyophilizing the pumpkin paste concentrate. The yield of the lyophilized sample was 37.5 g from 100 g of the pumpkin paste concentrate and was used as pumpkin in the subsequent animal experiments. The pumpkin was mixed with a basal diet before use each day for the animal experiments over duration of 76 d.

Animals. Male Wistar and GK (GK/Slc) rats (8 weeks old) were purchased from Clea Japan, Tokyo, Japan. The rats were maintained with a 12:12 h light-dark cycle at $22 \pm 2^\circ\text{C}$ and 40–60% humidity. The diet and water were given *ad libitum*, and the body weight was measured every other day.

Experiment 1: After acclimatizing for 3 d, the GK rats were assigned 2 groups of 5 each, fed on either the basal diet (control group) or the basal diet containing pumpkin (pumpkin group). The composition of each experimental diet is shown in Table 1. The ratio of pumpkin in the diet was set at 1%, because an anti-hypertensive effect was observed on SHR fed on the basal diet containing 1% of pumpkin.⁷⁾

Experiment 2: After acclimatizing for 4 d, the GK rats were assigned 3 groups of 6 each, fed on either the basal diet (control (CON) group) or the basal diet containing trigonelline (TRG group) or nicotinic acid (NA group). TRG and NA were purchased from Sigma-Aldrich, Missouri, USA and Kanto Chemical Co., Tokyo, Japan, respectively. Wistar rats (5 rats) fed on the basal diet were used as normal rats without diabetes. The composition of each experimental diet is shown in Table 1. Equimolar amounts of TRG and NA (0.406 mmole) were added to the diets (0.056 and 0.05%, respectively). The diets were administered for 43 d.

Blood was collected by cardiac puncture from rats that had been anesthetized with Nembutal (Dainippon Pharmaceutical Co., Osaka, Japan) after 10 h of fasting on the last day for the feeding period of the experimental diet. Serum was prepared by centrifuging the collected blood at $1,000 \times g$ for 15 min. The liver was detached and stored at -80°C until needed for analysis. The left kidney and left epididymal adipose tissues were also detached and weighed.

The rats were cared for at all times according to the institutional guidelines of Yamagata University.

Isolation and identification of the active components. The edible portion of the pumpkin (5 kg) was homogenized with 5 volumes of methanol (MeOH), before being extracted for 3 h under reflux. The residue obtained by centrifugation of the extracted homogenate was again treated in the same way. The supernatants obtained were combined and concentrated, loaded into a silica gel column (5.5 i.d. \times 38 cm) that had been equilibrated with CHCl_3 :MeOH = 9:1 (v/v), and then successively eluted with CHCl_3 :MeOH = 9:1, 7:3, and 5:5 (v/v). The eluate obtained with CHCl_3 :MeOH = 5:5 was further

fractionated by preparative HPLC in an ODS C30-UG-5 column (20 i.d. \times 250 mm; Nomura Chemical Co., Aichi, Japan), using a solvent system composed of 5% CH_2CN in 1% acetic acid (A) and 40% CH_2CN (B) as 0–100% B in A, linear gradient. The flow rate and detection wavelength were set at 2.5 ml/min and 260 nm, respectively.

¹H- and ¹³C-NMR spectra were obtained in D₂O with a Jeol JNM-EX 400FT-NMR spectrometer. Chemical shifts are expressed as δ values. FAB-MS data were recorded with a Jeol JMX-D 300 spectrometer.

Assays of fasting and fed blood glucose levels, and blood glucose tolerance test. The fasting blood glucose level was measured after 10 h of fasting, and the fed blood glucose level was measured 1 h after dietary intake at the end of weeks 1, 3 and 5. The glucose levels were measured with a Medesafe GR-102 (Termo Co., Tokyo, Japan, 20–600 mg/dl measuring range).

The glucose tolerance test was carried out on rats that had been fasted for 10 h on day 49 of the feeding period for experiment 1, and on days 26–27 of the feeding period for experiment 2. The rats were administered with a 40% glucose solution (2 g/kg of body weight). Blood was collected from the tail vein 0, 15, 30, 60, 120 and 180 min after glucose loading, and the glucose levels were measured with a Medesafe GR-102 instrument.

Measurement of the insulin, adiponectin, and tumor necrosis factor (TNF)- α levels. The serum levels of insulin, adiponectin and TNF- α were measured with ELISA kits (Levis rat insulin kit, Shibayagi Co., Gunma, Japan; mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical Co., Tokyo, Japan; and rat TNF- α kit, BioSource International, California, USA).

Measurements of thiobarbituric acid reactive substances (TBARS) and hemoglobin A_{1c} (HbA_{1c}). For blood TBARS measurement, 0.1 ml of blood collected by cardiac puncture as already described was mixed with 1.9 ml of physiological saline, before being centrifuged at $1,000 \times g$ for 10 min. The TBARS content in the supernatant was measured according to the Yagi⁸⁾ method and is expressed as nmol malondialdehyde per ml of blood. The concentration of liver TBARS was measured by the method of Uchiyama and Mihara,⁹⁾ using a homogenate prepared by mixing 0.5 g of the frozen liver with 9 volumes of a cold 1.15% KCl solution.

HbA_{1c} was measured with a Micromat II instrument (Bio-Rad Laboratories, California, USA) after 1, 4 and 6 weeks of the feeding period. The principle of affinity chromatography method was used in the measurement to measure the percentage of glycated hemoglobin in the blood.

Measurement of 8-hydroxydeoxyguanosine (8-OHdG). To determine urinary 8-OHdG, urine collected for 40 h during days 58–59 was centrifuged at $1,000 \times g$ for 20 min, and the precipitate was discarded. The content of 8-OHdG in the supernatant was measured with an 8-OHdG Elisa kit (Japan Institute for the Control of Aging Co., Shizuoka, Japan)^{10,11)} and is expressed relative to the content of creatinine. The creatinine content was measured with a commercial kit (Creatinine-kit Wako, Wako Pure Chemical Industries, Osaka, Japan).

Lipid analysis. Total- and HDL-cholesterol (T-Chol and HDL-Chol), triglyceride (TG), phospholipid (PL), total non-esterified fatty acids (NEFA), and total bile acids were measured enzymatically with commercial kits (cholesterol E test, HDL-cholesterol test, triglyceride E test, phospholipid B test, NEFA C test and total bile acid test; Wako Pure Chemical Industries, Osaka, Japan). Lecithin, sphingomyelin and lysolecithin could be measured by using the phospholipid B test.

Liver total lipid was extracted by the method of Folch *et al.*¹²⁾ Five g of liver was homogenized with a mixture of 5 ml of MeOH and 10 ml of CHCl_3 . The homogenate was filtered, and the filtrate was added to one-fourth of its volume of 0.88% KCl to separate the chloroform layer. An aliquot of the chloroform layer was evaporated to measure the total lipid, and further dissolved in 1 ml of isopropanol for measuring the T-Chol, TG, PL and NEFA levels.

Liver enzyme activities. To measure the fatty acid synthase (FAS) activity, a liver homogenate was prepared by the Burton¹³⁾ method.

Table 1. Composition of the Diets (%)

Constituent	Experiment 1		Experiment 2		
	Basal	Pumpkin	Basal	Trigonelline	Nicotinic acid
Casein ¹	15	15	15	15	15
α -Cornstarch:sucrose = 2:1	69.5	68.5	70.5	70.44	70.45
Corn oil	5	5	5	5	5
Celulose	5	5	5	5	5
Vitamin mixture ²	1	1	1	1	1
Mineral mixture ³	3.5	3.5	3.5	3.5	3.5
NaCl	1	1			
Pumpkin		1			
Trigonelline				0.056*	
Nicotinic acid					0.05*

¹Since it is known that food rich in starch and sucrose enhances the risk of type 2 diabetes,⁴³⁾ the protein level was set to 15% instead of 20% (the level recommended in the AIN 96 diet) to increase the ratio of α -cornstarch+sucrose in the diet, expecting the earlier induction of severe type 2 diabetes in the GK rats.

²AIN-93G-MX and ³AIN-93-VX, which contained 25 g of bitartrate per 100 g, were obtained from Oriental Yeast Co.

*Equimolar trigonelline and nicotinic acid (0.406 mmole) were added to the respective diets.

The FAS activity was determined in terms of malonyl-CoA- and acetyl-CoA-dependent oxidation of NADPH according to the methods of Kumer *et al.* and Carey *et al.*^{14,15} Briefly, a liver sample was homogenized in a 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM mercaptoethanol. The homogenate was centrifuged at $1,000 \times g$ for 15 min at 4 °C, and the supernatant was further centrifuged at $100,000 \times g$ for 45 min at 4 °C. The enzyme activity of the supernatant was assayed in a 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM mercaptoethanol, 1 mM EDTA, 10 mM NADPH, 0.5 mM malonyl-CoA and 0.33 mM acetyl-CoA. The rate of decrease in absorbance at 340 nm was measured.

The glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) activities were measured with a Bioxytech G6PD/6PGD-340 kit (Oxis International, California, USA).

To measure the carnitine palmitoyl transferase (CPT) activity, a liver sample was homogenized in a 3 mM Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose and 1 mM EDTA. The reaction mixture was composed of a 58 mM Tris-HCl buffer (pH 8.0) containing 0.25 mM DTNB, 0.04 mM palmitoyl CoA, 1.25 mM EDTA and 1.25 mM L-carnitine, and the homogenate. The CPT activity was determined from the rate of change in absorbance at 412 nm.¹⁶

The glucokinase (GLK) activity was measured spectrophotometrically.¹⁷ A liver sample was homogenized in an ice-cold buffer (pH 7.5) containing 50 mM HEPES, 250 mM sucrose, 100 mM KCl, 1 mM EDTA, 5 mM MgCl₂, and 2.5 mM dithioerythritol, and the homogenate was centrifuged at $105,000 \times g$ for 60 min. The hexokinase activity of the supernatant was measured in a buffer (pH 7.4) containing 50 mM HEPES, 7.5 mM MgCl₂, 100 mM KCl, 5 mM ATP, 2.5 mM dithioerythritol, 10 mg/ml BSA, 0.5 mM NAD⁺, 4 U/ml of glucose-6-phosphate dehydrogenase (*L. mesenteroides*) and 0.5 mM glucose. The total phosphorylating activity was measured by using 100 mM glucose instead of 0.5 mM glucose. The reaction was initiated by adding ATP, and the rate of increase in absorbance due to NADH formation was recorded at 340 nm. The GLK activity was calculated as the difference between the total phosphorylating activity and hexokinase activity.

The glucose-6-phosphatase (G6Pase) activity was measured by using the microsomal fraction obtained as a precipitate by centrifugation at $105,000 \times g$. The microsomal fraction was re-suspended in a homogenization buffer and diluted with an ice-cold buffer (pH 6.5) containing 100 mM HEPES and 0.1 mM EDTA. The reaction was initiated by adding 10 mM glucose-6-phosphate at 37 °C, and stopped after 20 min by adding 2.2 volumes of a solution containing 3.7 mM ammonium molybdate and 240 mM SDS in 270 mM of H₂SO₄. After adding one-ninth of the volume of 1.2 M ascorbic acid, the reaction mixture was further incubated for 1 h at 37 °C, and the absorbance was measured at 820 nm.¹⁸

Statistical analysis. Each value is given as the mean \pm SEM. The homogeneity of variance between treatments was verified by Bartlett's test. Data were statistically analyzed by a one-way analysis of variance (ANOVA). A *post-hoc* analysis of significance was made by using Fisher's PLSD test, where differences were considered significant at $p < 0.05$.

Results

Experiment 1

Oral glucose tolerance test (OGTT)

The effects of pumpkin on the blood glucose tolerance determined after feeding the experimental diet for 49 d are shown in Fig. 1A. The blood glucose level 30 min after glucose loading was significantly lower in the pumpkin group than in the control group. The area under the curve (AUC) for OGTT is shown Fig. 1B, AUC for the pumpkin group also being significantly lower than in the control group.

Serum insulin and lipid levels

There was no statistically significant difference in serum insulin level between the control and pumpkin groups (Table 2).

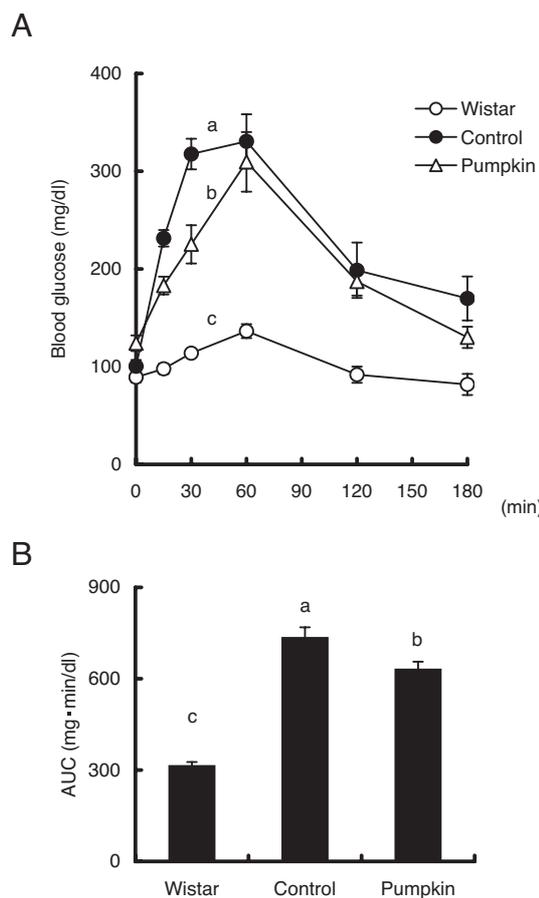


Fig. 1. Effect of Dietary Pumpkin on the Blood Glucose Level during the Oral Glucose Tolerance Test (OGTT) (Experiment 1).

A, OGTT was carried out on day 49 of the feeding period with GK and Wistar rats fed on diet with or without pumpkin; B, area under the curve (AUC) from OGTT. Values without a common letter at the same time point differ significantly ($p < 0.05$).

Table 2. Effects of Dietary Pumpkin on the Serum Insulin and Lipid Levels of GK Rats (Experiment 1)

Dietary group	Control	Pumpkin
Initial body weight (g)	142 \pm 4	138 \pm 5
Food intake (g/76 d)	799 \pm 17	812 \pm 10
Body weight gain (g)	191 \pm 7	196 \pm 3
Insulin (pg/ml)	75.6 \pm 0.4	76.5 \pm 0.9
Serum lipid		
T-Chol (mg/dl)	109 \pm 4	93.6 \pm 3.1*
HDL-Chol (mg/dl)	63.6 \pm 4.6	66.5 \pm 2.6
Atherogenic index ¹	0.74 \pm 0.09	0.41 \pm 0.04*
TG (mg/dl)	47.9 \pm 6.7	45.7 \pm 4.6
NEFA (mEq/l)	0.68 \pm 0.17	0.11 \pm 0.06*

¹(T-Chol-HDL-Chol) /HDL-Chol.

*Significantly different from the control group, $p < 0.05$.

Serum T-Chol and the atherogenic index were significantly lower in the pumpkin group than in the control group (Table 2). The TG level in the serum did not differ between the control and pumpkin groups, although compared to the control group, the NEFA level was significantly lower in the pumpkin group (Table 2).

Structural determination of the pumpkin constituents

Both MeOH extracts of the pumpkin paste concentrate and the edible portion of the pumpkin showed similar HPLC traces (Fig. 2). In this experiment, the compounds corresponding to peaks 1 and 2 in Fig. 2

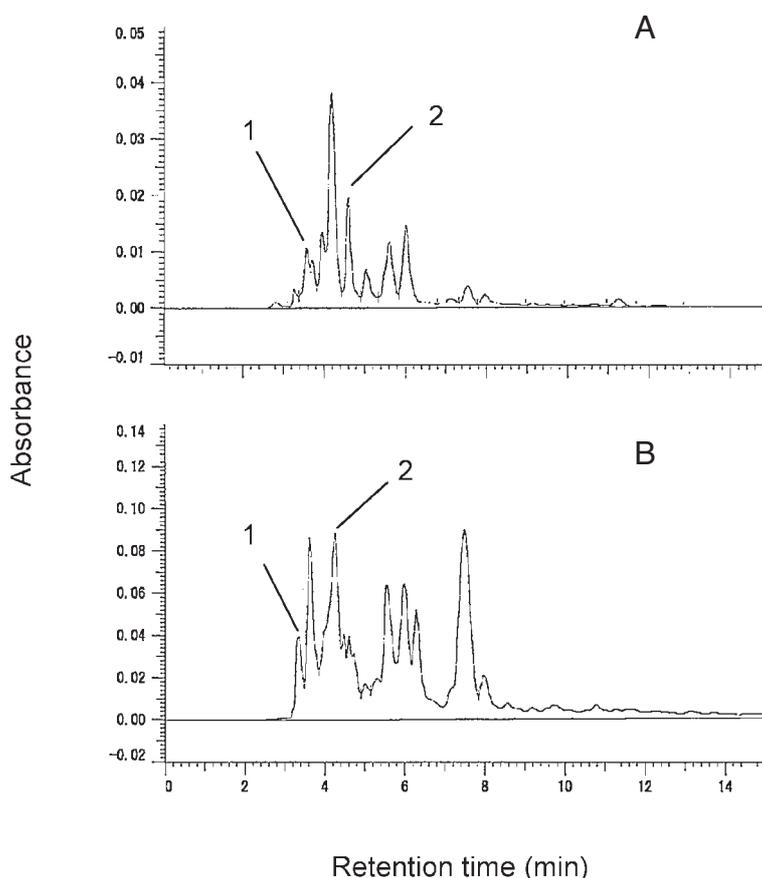


Fig. 2. HPLC Traces of the Pumpkin Paste Concentrate (A) and Pumpkin MeOH Extracts (B).

UV spectra of peaks 1 and 2 were similar to those of trigonelline and nicotinic acid, respectively. HPLC conditions: column, Develosil C-30-UG-5 (4.6 i.d. \times 250 mm, Nomura Chemical Co., Aichi, Japan); development, a linear gradient of 0–100% of solvent B (40% CH_4CN) in solvent A (5% CH_4CN in 1% acetic acid) over the course of 180 min at a flow rate of 0.8 ml/min; detection at 260 nm.

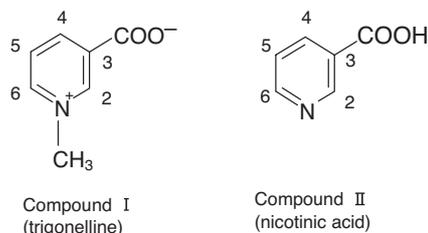


Fig. 3. Chemical Structures of Trigonelline and Nicotinic Acid.

(compounds I and II in Fig. 3) were isolated because they showed a UV spectral resemblance to that of nicotinic acid. Nicotinic acid has been reported to have improved atherogenic dyslipidemia,¹⁹ this being closely related to the induction of diabetes.^{20,21} FAB-MS data for compounds I and II exhibited pseudo molecular ions $[\text{M} + \text{H}]^+$ at m/z 138 and 124, respectively. The ^{13}C -NMR spectral data for the two isolated compounds showed good agreement with those of authentic trigonelline and nicotinic acid. δ_{C} (100 MHz, D_2O) for compound I: 49.0 (CH_3), 128.4 (C-5), 137.5 (C-3), 145.6 (C-4), 146.6 (C-2), 146.8 (C-6), 168.4 (C-7); δ_{C} (100 MHz, D_2O) for compound II: 128.3 (C-5), 136.9 (C-3), 143.8 (C-2), 144.4 (C-4), 147.3 (C-6), 169.7 (C-7). These spectral data enabled compounds I and II to be identified as TRG and NA, respectively. The amounts of TRG and NA in the edible part of the pumpkin were 12 and 210 mg per 100 g of fresh weight, respectively, as

determined by HPLC. The amounts of 11.2 and 10 mg of TRG and NA contained in 20 g of the diet given per day per rat in the TRG and NA groups in experiment 2 corresponded to amounts of about 93.3 and 4.76 g of pumpkin.

Experiment 2

Fasting and fed blood glucose levels, and OGTT

Although the fasting blood glucose levels did not differ among the control (CON), TRG and NA groups, the fed blood glucose levels after 3 and 5 weeks tended to be lower and significantly lower in the TRG group than in the CON group. On the other hand, the fed glucose level in the NA group tended to be lower only after 5 weeks (Fig. 4A and B).

The effects of TRG and NA on blood glucose tolerance, which was determined after feeding the experimental diet for 26–27 d, are shown in Fig. 4C. The blood glucose levels in the TRG and NA groups 15 and 30 min after glucose loading did not differ from those of the control group. However, those in the TRG group were significantly lower than in the control and NA groups 60 and 120 min after loading. AUC in OGTT is shown in Fig. 4D. The TRG group was also significantly lower than the control and NA groups, but AUC of the NA group did not differ from the control group.

Changes in the serum insulin level during OGTT are shown in Fig. 4E. At 0 min, just before glucose loading,

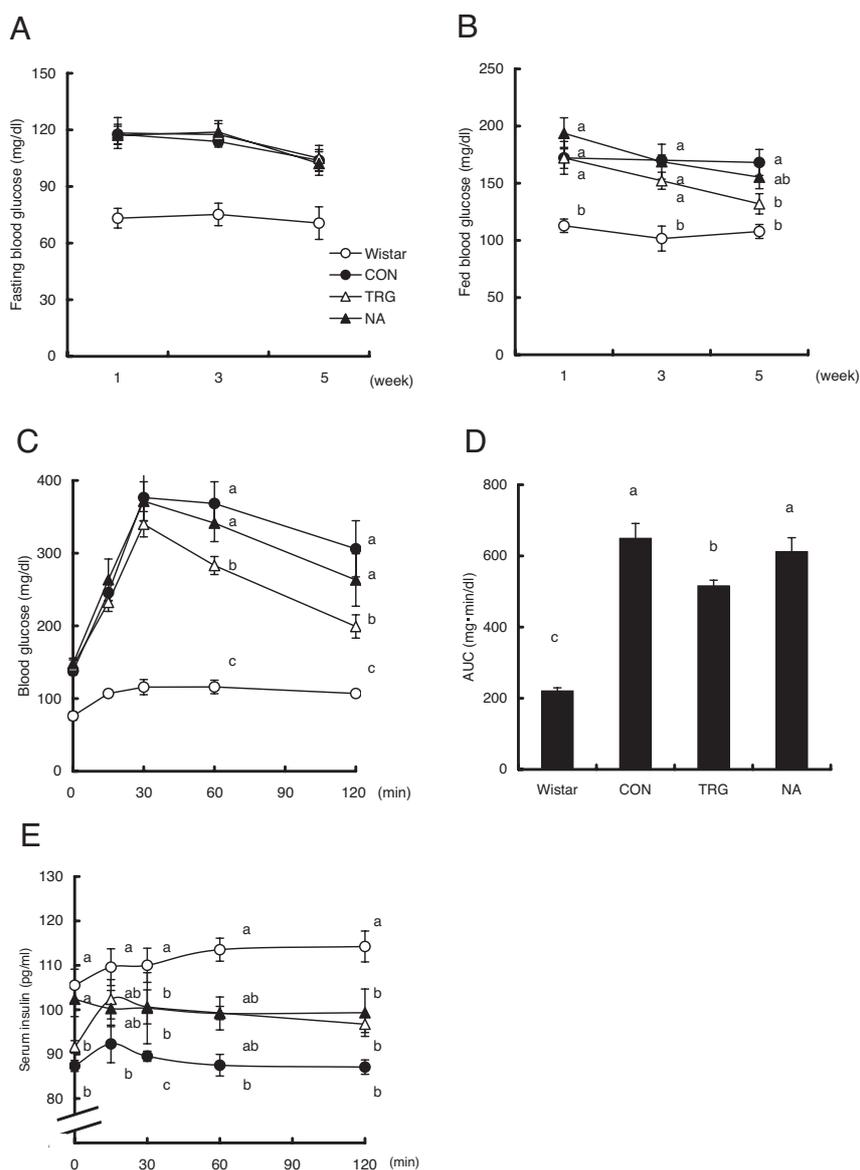


Fig. 4. Effect of Dietary Trigonelline and Nicotinic Acid on the Fasting (A) and Fed (B) Blood Glucose Levels, and on the Blood Glucose Level (C), AUC (D) and Insulin Levels (E) during the Glucose Tolerance Test.

A, fasting blood glucose was measured after 10 h of fasting at the end of weeks 1, 3 and 5; B, fed blood glucose was measured 1 h after dietary intake at the end of weeks 1, 3 and 5; C, glucose loading was carried out on days 26–27 of the feeding period; D, AUC (area under the curve); E, serum insulin concentration before and after glucose administration. Each values is mean \pm SEM ($n = 5$ for the Wistar group; $n = 6$ for the CON, TRG, and NA groups). Values without a common letter at the same week (A and B) and time (C and E) points, and among the dietary groups (D) differ significantly ($p < 0.05$).

the insulin level in the TRG group was significantly lower than in the control and NA groups; however these levels increased 15 min after glucose loading and then decreased slightly over 120 min. This phenomenon resembles that seen in the Wistar group. On the other hand, the insulin level in the control group increased gradually from 0 to 120 min. The insulin level in the NA group was unchanged over the 120 min period.

Body and organ weights

There were no significant differences in the total food intake and body weight gain among the 4 groups (Table 3). Although the liver weight did not differ among the control, TRG and NA groups, the kidney and epididymal adipose tissue weights were lower in the NA group than in the control group. The epididymal adipose tissue weight in the TRG group was also lower than that in the control group.

Serum insulin, adiponectin and TNF- α levels

The serum insulin level after the feeding period of 43 d was significantly lower in the TRG group than in the control and NA groups (Table 3). The adiponectin level did not differ among the 4 groups. The TNF- α levels in the TRG and NA groups were significantly lower and tended to be lower respectively, when compared with that of the control group.

Serum and liver lipid levels

The serum and liver lipid levels are shown in Table 4. The serum T-Chol, TG and NEFA levels were higher in the control group than in the Wistar group; however, those in the TRG and NA groups were lower than those in the control group. There were no statistical differences between the TRG and NA groups.

In the same way, the liver T-Chol, TG and NEFA levels were higher in the control group than in the

Table 3. Effects of Dietary Trigonelline and Nicotinic Acid on the Blood Glucose, Serum Insulin, Adiponectin and TNF- α Levels

Dietary group	Wistar	CON	TRG	NA
Initial body weight (g)	189 \pm 4	205 \pm 11	212 \pm 15	193 \pm 11
Food intake (g/43 days)	688 \pm 15	715 \pm 9	716 \pm 13	682 \pm 9
Body weight gain (g)	95.4 \pm 7.9	109 \pm 6	97.5 \pm 4.5	97.4 \pm 7.9
Liver weight (% of body weight)	3.19 \pm 0.06 ^b	3.44 \pm 0.09 ^a	3.32 \pm 0.05 ^a	3.27 \pm 0.07 ^a
Kidney weight (% of body weight)	0.31 \pm 0.01 ^c	0.42 \pm 0.01 ^a	0.41 \pm 0.01 ^{ab}	0.39 \pm 0.01 ^b
Epididymal adipose tissue (% of body weight)	0.75 \pm 0.05 ^b	1.02 \pm 0.06 ^a	0.69 \pm 0.04 ^b	0.74 \pm 0.02 ^b
Glucose (mg/dl)	82.4 \pm 10.0	111.8 \pm 2.3	116.0 \pm 2.7	104.4 \pm 9.7
Insulin (pg/ml)	87.2 \pm 0.4 ^c	116.9 \pm 4.9 ^a	103.7 \pm 3.4 ^b	118.5 \pm 6.9 ^a
Adiponectin (ng/ml)	96.3 \pm 6.4	82.4 \pm 25.8	92.8 \pm 19.1	100.9 \pm 2.4
TNF- α (pg/ml)	121 \pm 1 ^a	123 \pm 3 ^a	111 \pm 3 ^b	116 \pm 2 ^{ab}

Wistar and GK rats (14 weeks old) were each given the diet for 43 d. Each value is the mean \pm SEM, n = 5 for the Wistar and NA groups, n = 6 for the CON and TRG groups. Values without a common letter differ significantly ($p < 0.05$).

Table 4. Effects of Dietary Trigonelline and Nicotinic Acid on the Serum and Liver Lipid Levels

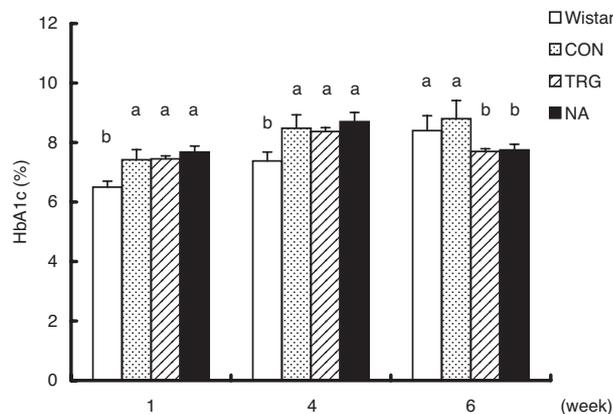
Dietary group	Wistar	CON	TRG	NA
Serum				
T-Chol (mg/dl)	66.0 \pm 5.8 ^b	123.3 \pm 21.5 ^a	87.7 \pm 2.9 ^b	83.6 \pm 2.0 ^b
HDL-Chol (mg/dl)	45.2 \pm 2.1 ^b	72.7 \pm 3.4 ^a	67.4 \pm 5.3 ^a	66.3 \pm 2.0 ^a
Atherogenic index ¹	0.45 \pm 0.08	0.68 \pm 0.27	0.33 \pm 0.09	0.46 \pm 0.02
TG (mg/dl)	82.2 \pm 5.3 ^a	90.9 \pm 16.6 ^a	43.8 \pm 4.6 ^b	45.3 \pm 2.8 ^b
NEFA (mEq/l)	0.63 \pm 0.04 ^b	0.70 \pm 0.05 ^a	0.57 \pm 0.06 ^b	0.72 \pm 0.11 ^b
Total bile acid (μ mol/l)	1.84 \pm 0.40 ^b	7.25 \pm 1.49 ^a	3.72 \pm 0.69 ^b	3.78 \pm 0.88 ^b
Liver				
Total lipid (mg/g of liver)	51.7 \pm 5.5 ^a	58.7 \pm 5.6 ^a	37.0 \pm 3.3 ^b	45.1 \pm 2.4 ^{ab}
T-Chol (mg/g of liver)	3.15 \pm 0.62 ^b	4.96 \pm 0.64 ^a	2.90 \pm 0.25 ^b	2.79 \pm 0.32 ^b
TG (mg/g of liver)	16.0 \pm 2.2 ^b	52.2 \pm 8.5 ^a	15.6 \pm 1.6 ^b	18.4 \pm 2.6 ^b
PL (mg/g of liver)	16.5 \pm 1.6	23.0 \pm 3.5	19.5 \pm 2.1	16.1 \pm 2.4
NEFA (mEq/g of liver)	65.1 \pm 1.4 ^{ab}	66.8 \pm 2.0 ^a	57.5 \pm 3.1 ^b	61.9 \pm 4.6 ^{ab}

¹(T-Chol-HDL-Chol)/HDL-Chol.

Wistar and GK rats (14 weeks old) were each given the diet for 43 d, and then the serum and liver lipid levels were measured.

Each value is the mean \pm SEM, n = 5 for the Wistar and NA groups, n = 6 for the CON and TRG groups.

Values without a common letter differ significantly ($p < 0.05$).

**Fig. 5.** Effects of Dietary Trigonelline and Nicotinic Acid on the Hemoglobin A_{1c} Level.

Unfilled, dotted, hatched and filled bars represent the HbA_{1c} levels for the Wistar, control, trigonelline and nicotinic acid groups. Values without a common letter at the same week point differ significantly ($p < 0.05$).

Wistar group. However, the rises in those values for the control group were limited, or tended to be limited, when feeding with TRG and NA.

HbA_{1c} levels

The HbA_{1c} levels measured after 1, 4 and 6 weeks of the feeding period are shown in Fig. 5. Although the HbA_{1c} levels after 1 and 4 weeks did not differ among the GK rats, the levels after 6 weeks were significantly lower in the TRG and NA groups than in the control group.

Liver FAS, G6PD + 6PGD and CPT activities

The activities of the liver FAS, G6PD + 6PGD and CPT enzymes are shown in Fig. 6. Only the TRG group showed significantly lower levels of FAS, and G6PD + 6PGD activities. On the other hand, the CPT activity tended to be higher in the TRG group and was significantly higher in the NA group when compared to the level in the control group.

TBARS and urinary 8-OHdG levels

The blood TBARS levels in the TRG and NA groups were significantly lower and tended to be lower, respectively, when compared to that of the control group (Table 5). In addition, the liver TBARS level in the TRG group was a significantly lower than that of the control group. The urinary excretion of 8-OHdG, a marker of oxidative stress, tended to be increased in the control group than in the Wistar group, although this tendency was absent in the TRG group. However, this phenomenon was not apparent in the NA group.

GLK and G6Pase activities

The activity of GLK, the rate-limiting enzyme on the glycolytic pathway, was significantly higher in the TRG group than in the control group, and that of the NA group tended to be higher than in the control group as well (Fig. 7A). No significant difference was apparent among groups in the activity of G6Pase, the enzyme which regulates gluconeogenesis. When the GLK/G6Pase ratios were compared, the ratio in the TRG group was significantly higher than that of the control

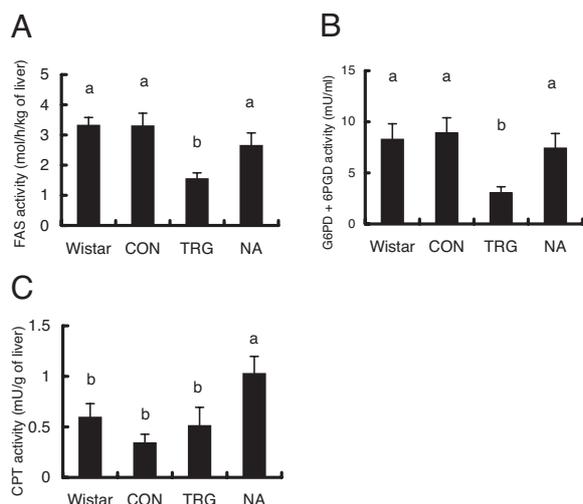


Fig. 6. Effects of Dietary Trigonelline and Nicotinic Acid on the Liver FAS, G6PD + 6PGD and CPT Activities.

A, liver fatty acid synthase (FAS) activity; B, summed activity of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD); C, carnitine palmitoyl transferase (CPT) activity. Each value is the mean \pm SEM ($n = 5$ for the Wistar and NA groups; $n = 6$ for the CON and TRG groups). Groups without a common letter differ significantly ($p < 0.05$).

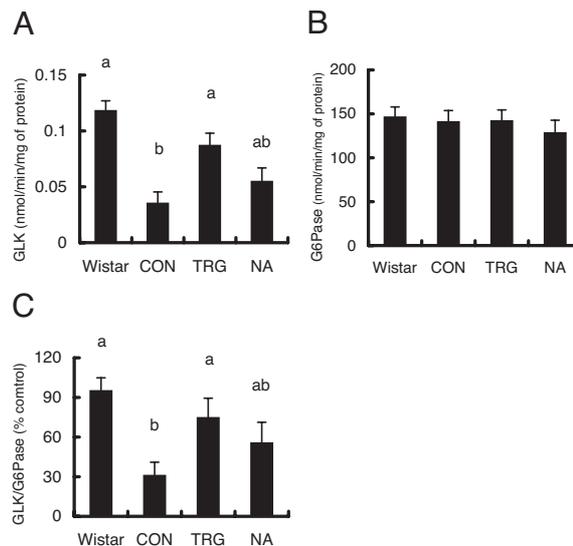


Fig. 7. Effects of Dietary Trigonelline and Nicotinic Acid on the Liver GLK and G6Pase Activities, and on the Activity Ratio of GLK to G6Pase (GLK/G6Pas ratio).

A, glucokinase (GLK) activity; B, glucose-6-phosphatase (G6Pase) activity; C, GLK/G6Pase ratio. Each value is the mean \pm SEM ($n = 5$ for the Wistar and NA groups; $n = 6$ for the CON and TRG groups). Values without a common letter differ significantly ($p < 0.05$).

Table 5. Effects of Trigonelline and Nicotinic Acid on the Oxidative Status

Dietary group	Wistar	CON	TRG	NA
TBARS				
Blood (nmol/ml)	1.32 \pm 0.35 ^{ab}	1.46 \pm 0.46 ^a	0.47 \pm 0.15 ^b	0.53 \pm 0.25 ^{ab}
Liver (nmol/g of liver)	172 \pm 15 ^{ab}	178 \pm 5 ^a	148 \pm 6 ^b	188 \pm 16 ^a
Urinary 8-OHdG (ng/g of creatinine)	442 \pm 85 ^a	665 \pm 158 ^a	239 \pm 59 ^b	727 \pm 185 ^a

Each value is the mean \pm SEM, $n = 5$ for the Wistar and NA groups, $n = 6$ for the CON and TRG groups. Values without a common letter differ significantly ($p < 0.05$).

group, and that of the NA group tended to be higher than that of the control group (Fig. 7C).

Discussion

It was demonstrated in experiment 1 that the diet containing the pumpkin paste concentrate improved the glucose tolerance and maintained a lower serum T-Chol level and atherogenic index compared to the control diet in the T2DM GK rat model (Fig. 1, Table 2). The decrease in blood glucose level after 30 min, together with decreased AUC on OGTT in the pumpkin group by 30 and 15%, respectively, compared to the values in the control group, suggest that the pumpkin contained compounds capable of mitigating the progression of diabetes. There was no difference in fasting insulin level between the control and pumpkin groups (Table 2), while there was a greater improvement in glucose tolerance in the pumpkin group as measured by OGTT (Fig. 1). This indicates that glucose tolerance was more readily affected by dietary pumpkin than the serum insulin levels in GK rats. Since it has been pointed out that a lower NEFA level was linked to a lower serum glucose level and improved insulin resistance,^{22,23} the lower NEFA level in the pumpkin group than in the control group may also support the possibility that the pumpkin paste concentrate had the ability to improve diabetes.

It has been reported that nicotinic acid was an effective agent for improving atherogenic dyslipidemia,¹⁹ and that it decreased the TG and apolipoprotein B levels in very low-density lipoproteins and increased high-density lipoprotein cholesterol. It has also been reported that the decreased in TG in very low-density lipoprotein was closely related to the inhibition of NEFA release from adipose tissue.²⁴ Furthermore, it is known that NA, a G protein agonist in fat cells, down-regulates cAMP and inhibits the activation of hormone-sensitive lipase, resulting in the induction of dyslipidemia and subsequent insulin resistance.^{20,21} These reports may indicate that a part of the improved glucose tolerance from pumpkin in experiment 1 might have been due to NA and its related compounds. However, it is necessary to take into consideration the involvement of such other components as polysaccharides and β -carotene for the improved glucose tolerance. Thus, NA and its structurally related compounds, TRG, which was found in a relatively abundant amount in the HPLC chromatogram of pumpkin paste concentrate, and the MeOH extract of the edible portions of the pumpkin were each isolated, and their chemical structures were confirmed by NMR and MS analysis. Since the ability of these two compounds to improve diabetes had not previously been compared in non-obese type 2 diabetic rats such as the GK model, the preventive effects of

these compounds on the progression of diabetes were determined in relation to their chemical structures in the GK rats by measuring diabetes markers and the markers of diabetes-induced oxidative stress.

Although the fasting blood glucose levels did not differ among the control (CON), TRG and NA groups, the fed blood glucose level after 5 weeks was significantly lower in the TRG group than in the control group, while that of NA group did not differ from the control group (Fig. 4A and B). These results suggest that TRG was effective in improving the blood glucose level under hyperglycemia followed by feeding. When OGTT was carried out on day 43 of the feeding period, the control rats, in comparison with the non-diabetic Wistar rat strain, showed a higher blood glucose level over 30 min and higher insulin level over 120 min. These results indicate that the control rats were insulin resistant at this point (Fig. 4C and E). The significant although weak suppression (by dietary TRG and NA, respectively) of the increase in blood glucose level observed after 60 and 120 min in OGTT (Fig. 4C) indicate that TRG, which is a precursor of NA *in vivo*,²⁵ may be superior in improving glucose tolerance. On the other hand, the lower insulin level observed in the TRG group than in the NA group at time 0 of OGTT (Fig. 4E) may indicate that TRG was more effective than NA in improving the insulin resistance as well as the glucose tolerance. In addition, it has been reported that although NA was effective in improving the blood glucose level, this effect was weak.²⁶ However, the effects of TRG and NA on glucose tolerance and insulin resistance have not been compared before now.

Although the body weight gain by day 43 and the food intake during the feeding period did not differ among the groups, the epididymal adipose tissue weight in the TRG and NA groups was lower than that of the control group (Table 3). This suggests that TRG and NA inhibited lipid accumulation or promoted lipid β -oxidation. The serum adiponectin level, which is known to be decreased in diabetes,^{27,28} did not differ significantly among the four groups. However the TNF- α level, which is known to change in inverse proportion to the change in adiponectin level in regulating diabetes,^{27,28} was significantly lower in the TRG group and tended to be lower in the NA group than in the control group (Table 3). These results, together with the lower serum insulin level observed in the TRG group compared to the levels in the control and NA groups (Fig. 4E), also provide evidence that TRG was more effective than NA in suppressing the progression of diabetes.

It is known that reactive oxygen species (ROS), which are by-products of the progressive glycation (amino-carbonyl reaction) characteristic of diabetes,^{29–31} induce oxidative stress followed by lipid peroxidation. Therefore, the effects of TRG and NA on the formation of malondialdehyde, as an end-product of lipid peroxidation, and on the urinary excretion of 8-OHdG as a marker of DNA damage by ROS were also measured. The lower blood TBARS level and lower amount of urinary 8-OHdG in the TRG group than in the control group (Table 5) may indicate the ability of TRG to offset an increase in oxidative stress due to the progression of diabetes. It is known that NA is a stronger radical scavenger than TRG against hydroxyl,

nitric oxide and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals;³² however, the blood TBARS and urinary 8-OHdG levels tended to be lower in the TRG group than in the NA group. An inverse relationship between the order of magnitude in radical scavenging activity and of the *in vivo* effects of TRG and NA may need to be examined in the future.

Although the HbA_{1c} level in the Wistar and control groups each showed an almost linear increase from 1 to 6 weeks, that in the TRG and NA groups, which each showed an almost linear increase from 1 to 4 weeks and then to decreased to 6 weeks, was significantly lower than that of the control group at 6 weeks (Fig. 5). These results suggest that feeding TRG and NA over longer periods might be effective for mitigating diabetes. It is known that the HbA_{1c} level in diabetic C57BL/KsJ-db/db mice reflects the state of erythrocytes influenced by the diet that has been fed in the previous 2–3 weeks.³³ Therefore, the lower level of HbA_{1c} in the TRG and NA groups in comparison with the control group at 6 weeks suggests that dietary TRG and NA inhibited the glycation of hemoglobin, especially in the latter period of feeding.

Although the serum TG and NEFA levels, along with the liver TG level in both the TRG and NA groups were lower than those of the control group, no difference in the TG and NEFA levels between these two groups was apparent (Table 4). It can be inferred from this result that the effects of TRG and NA on the lipid level or its metabolism were almost independent of the difference in chemical structures between TRG and NA, despite the fact that the glucose tolerance was improved more effectively by TRG than by NA (Fig. 4). This result may also indicate that the chemical structure of positively charged TRG with a methyl group lacking in NA was important to the improved glucose tolerance. It also suggests that TRG, which is converted to NA *in vivo*,^{25,34–38} may be more effective for improving diabetes. Bakhiya *et al.* have reported that a minute structural difference (the presence or absence of an additional methyl group in an alkylated four-ring polycyclic hydrocarbon) could strongly affect the interaction with transporter proteins and direct the excretion route.³⁹ Therefore, part of the difference in the effects of TRG and NA may have been due to the difference in their transfer *in vivo* and/or metabolism.

Serum and liver TG and NEFA levels that are reportedly down-regulated in the process of improvement of diabetes (T2DM) are closely related to the regulation of glucose uptake or gluconeogenesis.^{21,22,40} Therefore, the significant lowering of TG by TRG and NA observed in this experiment may also indicate that TRG and NA are antidiabetic compounds.

The atherogenic index, which is known to be increased in type 2 diabetes mellitus (T2DM) with insulin resistance and hyperlipidemia,⁴¹ tended to be lower in the TRG- and NA-fed rats than in the control rats, indicating that TRG and NA may be effective to mitigate the arteriosclerosis involved with T2DM. In spite of that, the serum high-density lipoprotein cholesterol (HDL-Chol) level did not differ among the TRG, NA and control groups (Table 4). The lower serum total cholesterol level in the TRG and NA groups, compared to the control group, may indicate an increase in the

very low-density lipoprotein cholesterol (VLDL-Chol) and/or low-density lipoprotein cholesterol (LDL-Chol) level. It also indicates that these are closely related to T2DM as have been reported by Adeli *et al.*⁴²⁾ and that TRG and NA are effective in suppressing the increase in VLDL- and HDL-Chol levels induced by diabetes. However, the precise mechanism for the suppression of VLDL- and LDL-Chol levels by feeding with TRG and NA needs to be determined in future.

The lower levels of FAS and G6PD + 6PGD activities, and the higher ratio of GLK to G6Pase in the liver of the TRG group in comparison to the control group (Figs. 6A, B and 7C) suggest that the reduction in fatty acid synthesis and enhanced glycolysis by TRG were closely related to its activity of mitigating increases in the serum and liver TG levels, and the progression of diabetes. The effect of NA on each of these markers was weaker than that of TRG. On the other hand, as the liver CPT activity in the NA group was significantly higher than that in the control group, it is considered that the enhanced β -oxidation by NA was closely related to its stronger activity in suppressing at increase in TG level. However, differences between TRG and NA in their effects on the TG level and action mechanism remain to be investigated.

The results of this study demonstrate that dietary pumpkin was effective for improving glucose tolerance in the T2DM GK rat model, and that TRG and NA contained in pumpkin improved the glucose tolerance, accompanied by suppression of the increase in HbA_{1c} level and oxidative stress *in vivo*. Furthermore, these effects were more pronounced in the case of TRG than in NA. It has also been demonstrated that dietary TRG and NA suppressed increases in the serum and liver TG levels, presumably through down-regulation of liver FAS, and up-regulation of GLK and CPT activities. These results suggest that the regulation of these enzymes by TRG and NA may play a crucial role in mitigating the progression of diabetes in GK rats, although the precise mechanism involved remains to be determined.

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