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ORIGINAL CONTRIBUTION

Grapefruit juice improves glucose intolerance in streptozotocin-induced diabetes by suppressing hepatic gluconeogenesis

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Abstract
Purpose Hypoglycemic effects of grapefruit juice (GFJ) are widely recognized, but the mechanism(s) by which GFJ lowers blood glucose levels have not previously been investigated.
Methods Wistar rats [250–300 g body weight (BW)] were divided into eight groups ($n = 7$). Group 1 animals were orally treated with 3.0 ml/kg BW of distilled water for 60 days, while groups 3, 4, 5, 6 were similarly treated with 3.0 ml/kg BW of GFJ. Groups 4 and 7 as well as 2, 5, 6 and 8 were given 45.0 and 60.0 mg/kg BW intraperitoneal injections streptozotocin, respectively, while groups 2 and 6 animals were additionally injected with insulin (4.0 units/kg, S.C., b.d), respectively. Fasting blood glucose (FBG) and glucose tolerance tests were done. Hepatic glycogen content and glucokinase, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) activities were measured in homogenized liver tissues.
Results Diabetic rats, groups 2 and 4–8 exhibited significantly reduced weight gain but increased polydipsia compared to controls. FBG was significantly increased in diabetic rats compared to controls but were significantly improved in GFJ-treated—compared to non-treated—diabetic rats. Similarly, diabetic rats showed significant glucose intolerance compared to controls which was improved by GFJ treatment. GFJ treatment did not improve fasting plasma insulin in diabetic rats. GFJ treatment significantly elevated glucokinase activity and hepatic glycogen concentrations but suppressed the activities of G6Pase and PEPCK, respectively, in diabetic animals.
Conclusion These findings show that GFJ is not insulinotropic but improves glucose intolerance in diabetic rats by suppressing hepatic gluconeogenesis.

Keywords Grapefruit juice · Diabetes · Insulin · Gluconeogenesis

Introduction

The grapefruit has traditionally been liberally incorporated into many diets as an anti-obesity ingredient [1] and has also been associated with positive health benefits since ancient times. However, consumption of GFJ is also notoriously associated with drug interactions as it appears to increase plasma concentrations of many prescription medications [2–7]. These reports have largely impacted negatively on the realization of potential health-promoting medicinal benefits of GFJ despite the fact only a few cases of life-threatening or fatal adverse effects arising from GFJ–drug interactions have been reported.

Studies by Gorinstein et al. [8] have previously suggested that diets supplemented with grapefruit improve plasma lipid levels and increase plasma antioxidant activity in experimental animals. Similar studies conducted on human subjects have shown that red grapefruit significantly lowers serum total cholesterol, low-density lipoprotein cholesterol and triglycerides in patients with coronary atherosclerosis [9]. A recent study by Fujioka et al. [10] reported that consumption of whole grapefruit or GFJ is associated with significant weight loss and improved insulin resistance in patients with metabolic syndrome, compared to placebo. We recently reported that consumption of GFJ improves

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glucose tolerance in non-diabetic rats [11]. However, the mechanisms by which grapefruit juice or some of its bioactive chemical constituents cause(s) hypoglycemia have not been previously investigated.

Aims and objectives

1. To determine the effect of GFJ on blood glucose homeostasis in diabetic rats.
2. To investigate the effects of GFJ on the activities of key regulatory enzymes in hepatic glucose disposition in vivo.

Materials and methods

Chemicals and reagents

The following chemicals and reagents were purchased from Sigma-Aldrich Pty. Ltd (Johannesburg, South Africa): D-glucose, STZ, citrate and phosphate buffers, triethanolamine, EDTA, KOH, ethanol, anthrone, H_2SO_4, glucose-6-phosphatase, molybdic acid, sodium dodecyl sulfate (SDS), ascorbic acid, HEPES, KCl, dithiothreitol, $MgCl_2$, inositol diphosphate (IDP), NADH, phosphoenolpyruvate, Na_2CO_3, malate dehydrogenase, H_2SO_4.

Regular insulin, normal saline, portable glucometer and glucose test strips (Ascensia Elite®; Bayer AG, Leverkusen, Germany) were purchased from a local pharmacy. Halothane and other accessories were provided by the Biomedical Resource Unit of the University of KwaZulu-Natal, Durban, South Africa.

GFJ commercially processed from Ruby grapefruit (with the following declared nutritive contents per 100 ml: energy, 190 kJ; protein, 0.6 g; carbohydrate, 10.0 g; total fat, 0.0 g; total dietary fiber, 0.4 g; sodium 0.0 g) was purchased from a local Woolworths Groceries Stores in Durban, South Africa. No preservatives or any other food additives were used in this preparation (as per manufacturer's declaration).

Animal treatment

Male Wistar rats (*Rattus novergicus,* 250–300 g body weight) were divided into eight groups ($n = 7$) and housed, with free access to standard commercial chow and drinking tap water ad libitum. The rats were maintained on a 12-h dark-to-light cycle of 08.00- to 20.00-h light in an air-controlled room (temperature 25 ± 2 °C, humidity 55 % ± 5 %) and were handled with humane care according to the guidelines of the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, which approved the study.

Group 1 animals (control) were orally treated daily with 3.0 ml/kg BW of distilled water for 60 days, while groups 3, 4, 5, 6 were similarly treated with 3.0 ml/kg BW of GFJ (Table 1). Groups 4 and 7 were given single 45.0 mg/kg BW intraperitoneal injections of streptozotocin (STZ) dissolved in 0.2 ml of 0.1 M citrate buffer, pH 4.5, after an overnight starvation, while groups 2, 5, 6 and 8 were similarly given 60.0 mg/kg BW intraperitoneal injections of STZ. Blood glucose concentrations were determined in groups 2, 4, 5, 6, 7 and 8 by tail pricking 3 days after STZ injections to determine development of diabetes. Rats with random blood glucose levels more than 11.0 mmoles/L were deemed to be diabetic and therefore included in the study. Groups 2 and 6 animals were additionally treated with 4.0 IU/kg BW (subcutaneously, twice daily) of insulin Actraphane® HM which is biphasic for short- and long-term glycemic control, respectively (Table 1). Animal weights and water consumption were recorded daily.

All the animals were sacrificed at the end of the study by halothane overdose and blood samples collected by

Table 1 Animal treatment protocol. STZ (streptozotocin); Ins-D60 (insulin + STZ 60 mg/kg BW); GFJ-ND (GFJ-non-diabetic); GFJ-D45 (GFJ + STZ 45 mg); GFJ-D60 (GFJ + STZ 60 mg/kg BW); GFJ-Ins-60 (GFJ + Ins + STZ 60 mg/kg BW); D45 (STZ 45 mg/kg BW); D60 (STZ 60 mg/kg BW)

Groups	Designation	Treatment				
		Distilled H_20 (ml, p.o).	GFJ (ml/kg, p.o.)	Insulin (U/kg SC)	STZ (mg/kg, SC)	Glucose (g/kg, IP)
1	Control	3.0				3.0
2	Ins-D60			4.0	60.0	3.0
3	GFJ-ND		3.0			3.0
4	GFJ-D45		3.0		45.0	3.0
5	GFJ-D60		3.0		60.0	3.0
6	GFJ-Ins-D60		3.0	4.0	60.0	3.0
7	D45				45.0	3.0
8	D60				60.0	3.0

cardiac puncture for plasma insulin analysis. Rat livers were excised, rinsed in normal saline and snap frozen in liquid nitrogen and stored at −180 °C for further analysis of hepatic enzymes and glycogen content.

Methods

Blood glucose testing

Fasting blood glucose (FBG) tests were done on treatment days 0 and 59, whereas glucose tolerance tests (GTT) were done on day 59 and blood glucose concentrations analyzed by a portable glucometer after tail pricking. Animals in all treatment groups were starved overnight prior to GTT and FBG. GTT was done by intraperitoneal injection of 3.0 g/kg BW of glucose in normal saline, and blood glucose concentrations monitored thereafter at 0, 15, 30, 60 and 90 min. Areas under the curve (AUC) were calculated from blood glucose–time curves and presented as AUC units (mM × minutes).

Fasting plasma insulin

Plasma insulin levels were analyzed by ultrasensitive rat insulin enzyme-linked immunoassay kit (DRG Diagnostics, Marburg, Germany) as per the manufacturer's manual.

Liver glycogen content

Hepatic glycogen content was measured as per the modified method of Seifter et al. [12]. The liver tissues were homogenized in 5 volumes of an ice-cold 4.0 M KOH solution and dissolved in a boiling water bath (100 °C) for 30 min. The glycogen was then precipitated with ethanol, pelleted, washed and resolubilized in distilled water. The concentrations of glycogen in the liver tissues were then assayed by treatment with anthrone reagent (92 g/l anthrone in 95 % (v/v) H_2SO_4), and the absorbance measured at 620 nm. Glycogen content was expressed as mg/g liver protein.

Hepatic enzyme assays

Glucokinase activity

Liver samples that had previously been snap frozen were aliquoted 100 mg per tube and homogenized in buffer containing 50 mM HEPES, 100 mM KCl, 2.5 mM dithiothreitol, 1 mM EDTA and 5 mM $MgCl_2$. Homogenates were centrifuged at 100,000 g for 1.0 h at 4 °C, to sediment the microsomal fractions (which were kept for glucose-6-phosphate assay). The postmicrosomal supernatant was used for the spectrophotometric measurement as per the previously described methods of Davidson and Arion [13], and Barzila and Rosetti [14]. Total protein content was determined by Bradford method [15]. The formation of glucose-6-phosphate from glucose was coupled to oxidation by glucose-6-phosphate dehydrogenase and NAD^+ in a continuous reaction mix containing 50 mM HEPES, 100 mM KCl, 2.5 mM dithiothreitol, 7.5 mM $MgCl_2$, 5 mM ATP, 10 mg/ml albumin, glucose (0, 5, 10, 15, 25, 50 mM, respectively), 0.5 mM NAD^+, 4 units of glucose-6-phosphate dehydrogenase (from *Leuconostic mesenteroides*) and the equivalent of 1 mg of liver wet weight. The reaction was initiated by the addition of ATP, and the rate of NAD^+ reduction recorded at 340 nm for 30 min at 37 °C, using Beckman DU-70 spectrophotometer equipped with temperature controller. The enzyme activity was expressed as µmol/g liver protein/min.

Glucose-6-phosphatase (G6Pase) activity

Liver samples that had previously been snap frozen were similarly aliquoted 100 mg per tube and homogenized in buffer containing 50 mM HEPES, 100 mM KCl, 2.5 mM dithiothreitol, 1 mM EDTA and 5 mM $MgCl_2$. Homogenates were centrifuged at 100,000 g for 1.0 h at 4 °C, to sediment the microsomal fraction. Liver content of glucose-6-phosphatase content was measured spectrophotometrically as per the modified method of Lange et al. [16]. Total protein content was determined by Bradford method [15]. The microsomal fractions were incubated with 0, 0.5, 1.0, 2.5, 5 and 10 mM glucose-6-phosphate. The reaction was carried out at 37 °C and stopped after 30 min with a solution containing acid molybdate, with 2/9 volumes of 10 % SDS and 1/9 volume of 10 % ascorbic acid. The reaction mixture was then incubated at 45 °C for 20 min, and the absorbance read at 820 nm, using Beckman DU-70 spectrophotometer equipped with temperature controller. The assay was based on the hydrolysis of glucose-6-phosphate by tissue microsomal fraction containing glucose-6-phosphatase. A standard curve was constructed using different concentrations of free phosphate, and the enzyme activity was expressed as µmol/min/g of liver protein.

Phosphoenolpyruvate carboxykinase (PEPCK) activity

PEPCK activity was determined as per the modified methods of Bentle and Lardy [17] and Stiffin et al. [18]. Cytosolic fractions were obtained from homogenized liver tissues by centrifugation at 100,000 g for 1.0 h at 4 °C, and the activities of enzymes measured in a final reaction volume of 1.0 ml, at pH 7.0, containing 50 mM sodium HEPES/KOH buffer, 10.0 mM IDP, 1.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.25 mM NADH, 2.0 mM phosphoenolpyruvate, 50 mM Na_2CO_3 and 10 U of malic

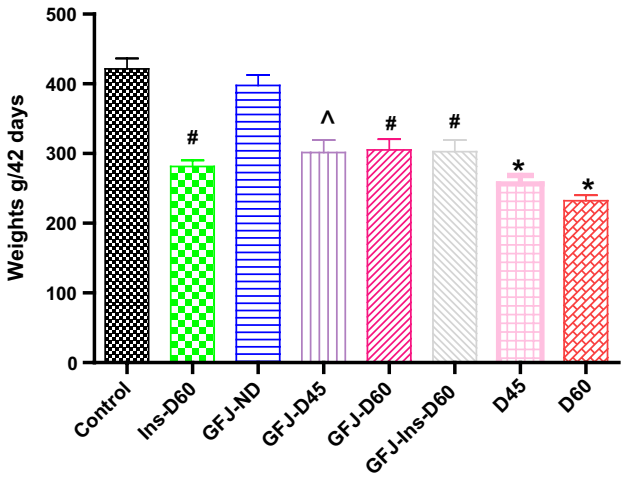

Fig. 1 Differences in animal weights before and after treatment. The animal weights were recorded daily; then, pre-treatment weights were subtracted from the final live weight at 42 days of treatment. $^{*}p < 0.0001$; $^{\#}p = 0.0036$; $^{\Lambda}p < 0.03$ compared to controls, D60 and D45, respectively

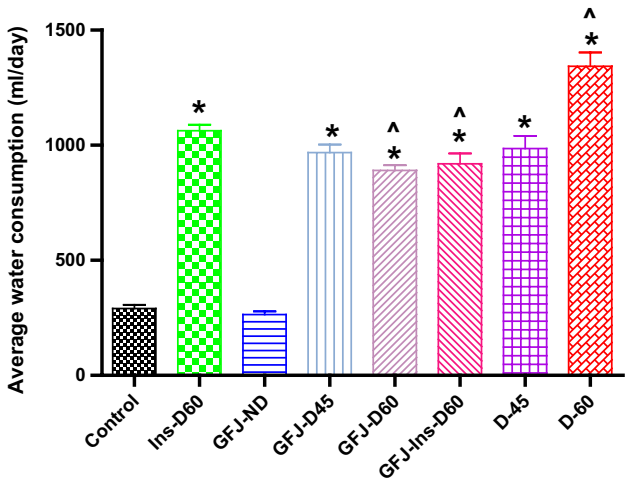

Fig. 2 Average daily water consumption in all treatment groups. The volume of water in drinking bottle was measured each morning before a fresh one was added. $^{*,\Lambda}p < 0.0001$ compared to controls and untreated diabetics, respectively

dehydrogenase (1 unit defined as 1 µmol of malate produced/min/mg of liver protein). Total protein content was determined by Bradford method [15]. All assay components were pre-incubated for 3 min. The enzyme activity was measured at 25 °C and 340 nm, using Beckman DU-70 spectrophotometer equipped with temperature controller, and expressed as mmol of oxaloacetate (OAA) formed/min/g of liver protein.

Statistical analysis

The data are presented as means with standard deviation (SD) and analyzed by Graphpad Prism software version 5.0. Mann–Whitney tests and/or Student's *t* tests were applied to the results to determine statistical significance. Values of $p < 0.05$ were taken to imply statistical significance.

Results

Fasting blood glucose, GTT and liver glycogen

Diabetic rats exhibited significantly ($p < 0.05$) reduced weight gain compared to controls; however, treatment with either insulin or GFJ significantly improved weight in diabetic animals (Fig. 1). Average daily water consumption was significantly ($p = 0.0001$) increased in all diabetic groups, but treatment with GFJ either alone or in combination with insulin significantly ($p = 0.0001$) improved water intake in diabetic rats (Fig. 2).

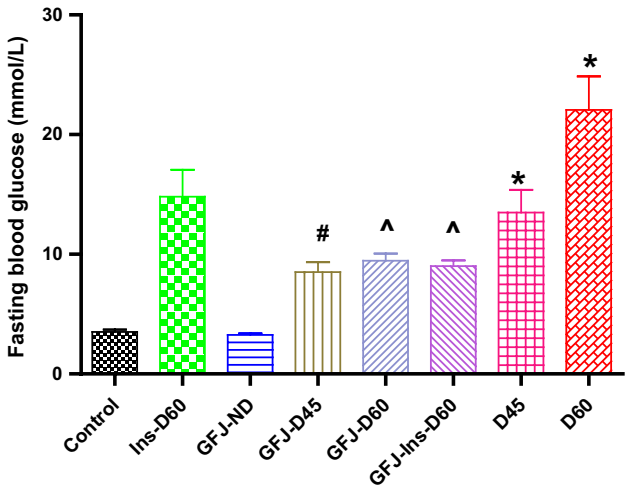

Fig. 3 Fasting blood glucose concentrations on day 59 of the treatment were measured by tail pricking and a portable glucometer after an overnight fast. Insulin treatment was withheld prior to the test. $^{*}p < 0.0001$; $^{\#}p = 0.045$; $^{\Lambda}p = 0.007$ compared to controls, untreated diabetics (D45) and D60, respectively

Fasting blood glucose concentrations were similar between GFJ-treated non-diabetic and control groups but were significantly ($p = 0.0001$) elevated in all diabetic groups compared to controls, respectively (Fig. 3). Treatment with GFJ either alone or in combination with insulin significantly ($p < 0.05$) reduced FBG levels in diabetic rats. Similarly, diabetic rats exhibited impaired glucose tolerance in GTT and calculated AUCs (Fig. 4) showed that treatment with GFJ alone or in combination with insulin significantly ($p < 0.05$) improved glucose intolerance in diabetic rats.

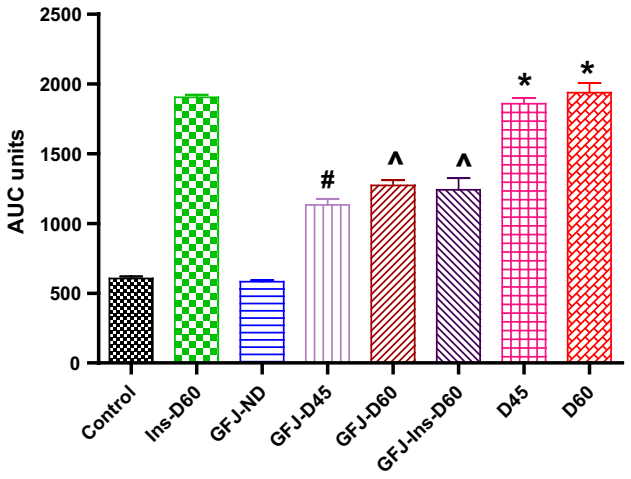

Fig. 4 AUCs calculated from glucose tolerance tests (GTTs) which was done after an overnight fast following IP injection of 3.0 g/kg BW of glucose in saline. Blood glucose concentrations were measured at 0, 15, 30, 60 and 90 min. $^{*}p < 0.0001$; $^{\#}p = 0.0012$; $^{\Lambda}p = 0.0034$ compared to controls, untreated diabetics (D45) and D60, respectively

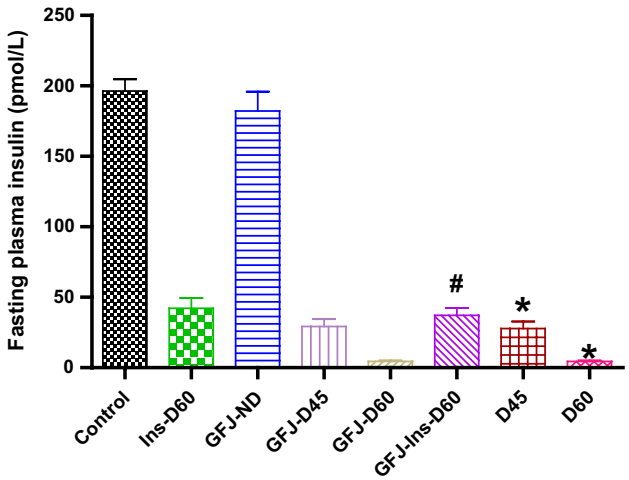

Fig. 5 Fasting plasma insulin concentrations. Insulin treatment was withheld prior to blood collection. Fasting plasma insulin was measured in blood drawn by cardiac puncture after halothane overdose. $^{*}p < 0.0001$; $^{\#}p = 0.005$ compared to controls and untreated diabetics (D60), respectively

Fasting plasma insulin levels were similar between controls and GFJ-treated non-diabetic rats but significantly ($p < 0.0001$) reduced in diabetic animals (Fig. 5). Rats that were treated with 45.0 mg/kg BW of STZ (D45) had significantly elevated fasting plasma insulin compared to the ones that were treated with 60.0 mg/kg BW of STZ (D60) (Fig. 5). GFJ treatment did not significantly elevate fasting plasma insulin concentrations in diabetic rats compared to non-treated ones. Insulin with GFJ significantly ($p < 0.05$) increased fasting plasma insulin in diabetic rats compared to the non-treated diabetic rats (D60).

Diabetic rats (D-60) had significantly ($p = 0.0328$) reduced hepatic glycogen content compared to controls; however, treatment with either GFJ alone or in combination with insulin significantly ($p = 0.024$) increased hepatic glycogen content in diabetic rats compared to non-treated diabetic rats (D-60) (Fig. 6). Interestingly, GFJ treatment significantly ($p = 0.00016$) increased liver glycogen concentrations in non-diabetic rats compared to controls.

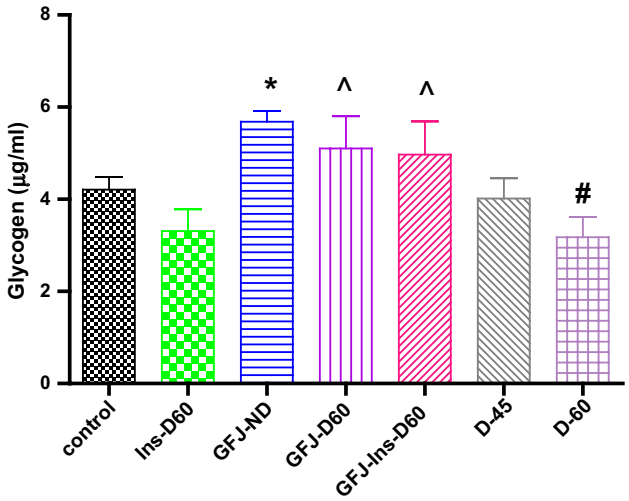

Fig. 6 Hepatic glycogen content was measured in homogenized liver tissues. $^{*}p = 0.0016$; $^{\#}p = 0.0328$; $^{\Lambda}p = 0.024$ compared to controls and untreated diabetics (D60), respectively

Liver enzymes

The activity of hepatic glucokinase obeyed Michaelis–Menten kinetics when plotted against substrate concentrations. Linear regression analysis of Eadie–Hofstee plots [13] showed that the activity of glucokinase was significantly ($p < 0.05$) reduced in diabetic (V_{max}; 14.8 (D45), 13.4 (D60) µmol/g of liver tissue/min, respectively) compared to control (V_{max}; 16.8 µmol/g of liver tissue/min) rats. However, treatment with either GFJ [V_{max}; 19.65 µmol/g of liver tissue/min (GFJ-D45), 14.5 µmol/g of liver tissue/min (GFJ-D60)], insulin [V_{max}; 20.3 µmol/g of liver tissue/min (Ins-D60)] or in combination with both [V_{max}; 19.4 ± 0.3 µmol/g of liver tissue/min (GFJ-Ins-D60)] significantly ($p < 0.05$) increased glucokinase activities compared to non-treated diabetic animals, respectively (Table 2). GFJ similarly increased glucokinase activity significantly ($p < 0.05$) in non-diabetic animals compared to controls.

The activity of hepatic G6Pase similarly obeyed Michaelis–Menten kinetics when plotted against substrate concentrations. When the data were transformed into

Table 2 Calculated glucokinase (Glck), G6Pase and PEPECK activities by Eadie–Hofstee plots

	Control Mean ± SD	Ins-D60 Mean ± SD	GFJ-ND Mean ± SD	GFJ-D45 Mean ± SD	GFJ-D60 Mean ± SD	GFJ-Ins-D60 Mean ± SD	D45 Mean ± SD	D60 Mean ± SD
Glck								
V_{max}	16.8 ± 0.4	20.3 ± 0.4	23.0 ± 0.9	19.7 ± 0.7	14.5 ± 0.3^	19.4 ± 0.3	14.8 ± 0.4	13.4 ± 0.3
km	0.27	0.37^{+}	0.21^{λ}	$0.36^{\#}$	0.35^	0.34^	0.28^{*}	0.28^{*}
G6Pase								
V_{max}	12.5 ± 0.3	18.0 ± 0.5	11.8 ± 0.3	16.7 ± 0.4	18.8 ± 0.3	17.6 ± 0.3	19.1 ± 0.3	22.6 ± 0.4
km	4.61	3.15^{+}	4.12	$2.92^{\#}$	5.13^	5.0^	6.37^{*}	4.52^{*}
PEPCK								
V_{max}	12.5 ± 0.3	18.0 ± 0.5	11.8 ± 0.3	16.7 ± 0.4	18.8 ± 0.3	17.6 ± 0.3	19.1 ± 0.3	22.6 ± 0.6
km	4.61	3.15^{+}	4.12	$2.92^{\#}$	5.13^	5.00^	6.37^{*}	4.520^{*}

Statistically significant differences are marked by relevant symbols applicable to different treatment groups: $^{*,\lambda}$ $p < 0.05$ compared to controls; $^{\wedge,+}$ $p < 0.05$ compared to untreated diabetic (D60); $^{\#}$ $p < 0.05$ compared to untreated diabetic (D45). V_{max} = (µmol/g of liver tissue protein/min)

Eadie–Hofstee plots [13], linear regression analysis showed that the activities of G6Pase were significantly ($p < 0.05$) elevated in diabetic [V_{max}: 19.1 µmol/min/g liver tissue; V_{max}/km: 121.8 (D45), 22.6 (D60) µmol/min/g liver tissue; V_{max}/km: 102.0 (D60)] compared to controls (V_{max}: 12.5 µmol/min/g liver tissue; V_{max}/km:53.39), respectively. However, in the presence of either GFJ [V_{max}; 16.7 ± 0.4 (D45), 18.8 (D60) µmol/min/g liver tissue; V_{max}/km: 48.80 (D45), 96.56 (D60)] or insulin (V_{max}: 18.0 µmol/min/g liver tissue, V_{max}/km: 56.62) alone or in combination with both (V_{max}: 17.6 µmol/min/g liver tissue, V_{max}/km: 87.89), G6Pase activity was significantly ($p < 0.05$) reduced in diabetic compared to non-treated diabetic (D45 and D60, respectively) animals. G6Pase activity in GFJ-treated non-diabetic rats (V_{max}: 11.8 µmol/min/g liver tissue; V_{max}/km: 48.48) was similar to that of the controls.

Similarly, PEPCK activity obeyed Michaelis–Menten kinetics when plotted against substrate concentrations. Linear regression analysis of Eadie–Hofstee plots [13] similarly showed significantly ($p < 0.05$) increased PEPCK activity in diabetic animals [V_{max}: 19.2 (D45), 22.2 ± 0.4 (D60) µmol/min/g liver tissue; V_{max}/km: 153.3 (D45), 119.2 (D60)] compared to controls (V_{max}: 13.9; V_{max}/km: 24.91). Treatment of diabetic rats with either GFJ [V_{max}: 16.8 (GFJ-D45), 17.0 (GFJ-D60) µmol/min/g liver tissue; V_{max}/km: 51.0 (GFJ-D45), 64.37 (GFJ-D60)] or insulin alone (V_{max}: 17.9 µmol/min/g liver tissue, V_{max}/km: 41.07) or in combination with both (V_{max}:17.9 µmol/min/g liver tissue; V_{max}/km: 71.62) significantly ($p < 0.05$) reduced PEPCK activity in diabetic rats compared to non-treated diabetic rats, respectively.

Discussion

In this study, models of types 2 and 1 diabetes were created in rats that were injected with 45 and 60 mg/BW of STZ, respectively. In the former group, there was partial but permanent β-cell destruction, while in the latter group, there was total β-cell destruction leading to near-absolute insulin deficiency. Consequently, diabetic rats exhibited reduced weight gain during natural growth as compared to controls (Fig. 1), but rats with partial β-cell destruction (D45) had slightly improved weight loss compared to those treated with 60 mg/BW of STZ (D60), suggesting that partial presence of insulin prevented accelerated lipolysis and proteolysis. However, treatment with either GFJ or insulin alone or in combination significantly improved weight loss in diabetic rats, respectively. This suggests that GFJ just like insulin may be inhibiting protein and lipid catabolism associated with insulin deficiency but did not affect the natural growth of non-diabetic rats (Fig. 1).

Diabetic rats experienced polydipsia as determined by water consumption (Fig. 2) but treatment with GFJ alone or in combination with insulin significantly reduced water intake compared to non-treated diabetic rats, while insulin alone did not reduce water intake. It may be possible that GFJ or some of its bioactive chemical constituents are protective against polydipsia in a diabetic state. Similarly, FBG concentrations were significantly increased in diabetic rats compared to controls, but treatment with either GFJ or insulin alone or combination reduced FBG concentrations in diabetic rats compared to non-treated diabetic animals, respectively (Fig. 3). Furthermore, diabetic rats presented with glucose intolerance compared to controls as determined by calculated AUC of blood glucose dose–response curves in GTT (Fig. 4). Consequently, treatment with either GFJ or insulin alone or in combination significantly improved glucose intolerance in diabetic rats compared to non-treated diabetic rats (Fig. 4).

Hypoglycemic effects of GFJ have previously been claimed [1, 10]. We have previously demonstrated blood glucose-lowering effects of GFJ in experimental

non-diabetic rats [11]. Diabetic patients often consume GFJ following folkloric claims of its anti-diabetic effects, and hence, to the best of our knowledge, this is the first time that anti-diabetic effects of GFJ have been demonstrated in diabetic animal models. However, the mechanisms by which GFJ could be exerting hypoglycemic effects have never been investigated.

We previously observed that GFJ does not influence pancreatic insulin secretion in non-diabetic experimental rats [11] and the current study further corroborates those findings as GFJ neither influenced insulin secretion in non-diabetic rats compared to controls nor increased plasma insulin concentration in diabetic animals (Fig. 5). This suggests that GFJ or its bioactive chemical constituents are neither insulinotropic nor promote pancreatic β-cell regeneration.

Our results suggest that GFJ increases hepatic glycogen concentrations both in non-diabetic and in diabetic (GFJ-D60) compared to controls and diabetic rats, respectively (Fig. 6). It is particularly interesting that GFJ significantly increased hepatic glycogen content in non-diabetic rats compared to controls suggesting that GFJ could be exerting a negative influence on the effects insulin counter-regulatory hormones such as glucagon, cortisol. Alternative GFJ could be reversing depletion of hepatic glycogen content in diabetic rats by activating mitogen-activated protein (MAP) kinase signaling pathways leading to phosphorylation of protein phosphatase-1, which in turn activates glycogen synthase and simultaneously deactivates glycogen phosphorylase, resulting in increased glycogen synthesis. Further studies are suggested to confirm this.

Negative correlation has recently been reported between hepatic glucokinase activity and severity of diabetes to the extent that reduced glucokinase activity leads to reduced glucose utilization with concomitant hyperglycemia [19]. In our study, glucokinase activity was significantly reduced in diabetic animals compared to controls (Table 2), while treatment of diabetic animals with either GFJ or insulin alone or in combination significantly improved glucokinase activity compared to non-treated diabetic animals, respectively (Table 2). However, it is noteworthy to point out that glucokinase activity was significantly elevated in diabetic rats treated with both GFJ and insulin compared to those that were treated with GFJ only, respectively (Table 2). This suggests that GFJ or its bioactive chemical constituents require insulin in the modulation of glucokinase activity. Increased glucokinase activity in response to GFJ treatment could partly explain the apparent increase in hepatic glycogen content in this group compared to the controls. Glucokinase catalyzes the rate-limiting step in the glycolytic pathway by phosphorylating glucose in both hepatocytes and pancreatic β cells. Rats lacking glucokinase activity in hepatocytes have previously been shown to be hyperglycemic and display significant defects in glycogen synthesis and glucose turnover rates [20]. Indeed, mutations in glucokinase gene in the pancreatic β cells are associated with the development of maturity onset diabetes of the young (MODY) in humans.

Diabetic animals displayed significantly increased G6Pase and PEPCK activities (Table 2), compared to controls, respectively, and treatment with either GFJ or insulin alone or in combination significantly reduced the activities of these enzymes in diabetic compared to non-treated diabetic rats, respectively. Both G6Pase and PEPCK regulate the rate-limiting steps in hepatic gluconeogenic flux and therefore contribute significantly to hyperglycemia in diabetes. Hepatic G6Pase catalyzes the hydrolysis of glucose-6-phosphate from glycogen in the last step of gluconeogenesis, while PEPCK converts oxaloacetate to phosphoenolpyruvate and carbondioxide, respectively [21].

Our study has not demonstrated whether GFJ increases the activity of glycolytic glucokinase leading to increased hepatic glycogen synthesis and simultaneously suppresses the activities of gluconeogenic enzymes by either increasing cellular protein content or gene transcription. Considering that GFJ treatment was chronic in these animals, and the similarity between GFJ and insulin effects, we cannot rule out enhancement of gene expression of these enzymes. As GFJ effects seem to be enhanced by insulin, we are tempted to speculate that GFJ, like metformin, could be upregulating the "master energy sensor," adenosine monophosphate protein kinase (AMPK), which is known to activate glucokinase and simultaneously deactivating G6Pase and PEPCK, respectively [22]. We have not demonstrated which chemical constituents of the GFJ are responsible for these anti-diabetic effects, but it is encouraging to note that anti-diabetic effects of flavonoids are well documented [23, 24]. A recent study by Jung et al. [25] showed that naringin and hesperidin, the main flavonoids in the GFJ, reduced blood glucose in diabetic mice by elevating hepatic glucokinase and glycogen concentration, by suppressing the activities of G6Pase and PEPCK, respectively. We chose to use GFJ as opposed to pure forms of isolated flavonoids since our previous experience [26] is that naringin does not exert hypoglycemic effects in type 1 diabetic model, suggesting that insulin is required for hypoglycemic effects of flavonoids and by extension GFJ as demonstrated in the present study.

Conclusion

This study demonstrates that hypoglycemic effects of GFJ are mediated in part by the regulation of the activities of key glycolytic and gluconeogenic enzymes in the liver and that insulin augments these effects. This suggests that GFJ or its bioactive chemical constituents are not insulinotropic

but like metformin require insulin in the regulation of glucose homeostasis. GFJ may therefore be beneficial to type 2 as opposed to type 1 diabetic patients. Further studies to investigate protein and lipid catabolism as well as precise molecular mechanisms underlying these anti-diabetic effects are suggested.

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Conflict of interest None to declare.

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