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Hypoglycemic Effects of a Phenolic Acid Fraction of Rice Bran and Ferulic Acid in C57BL/KsJ-*db/db* Mice

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Rice bran contains many phenolic acids, the most abundant of which is the antioxidant, ferulic acid (FA). We evaluated the hypoglycemic effects of a phenolic acid fraction (the ethyl acetate fraction, EAE) of rice bran and of FA in C57BL/KsJ *db/db* mice. Type 2 diabetic mice were allocated to a control group, an EAE group, or an FA group. Animals were fed a modified AIN-76 diet, and EAE or FA was administered orally for 17 days. There was no significant difference in body weight gain between groups. Administration of EAE and FA significantly decreased blood glucose levels and increased plasma insulin levels. EAE or FA groups had significantly elevated hepatic glycogen synthesis and glucokinase activity compared with the control group. Plasma total cholesterol and low density lipoprotein (LDL) cholesterol concentrations were significantly decreased by EAE and FA administration. These findings suggest that EAE and FA may be beneficial for treatment of type 2 diabetes because they regulate blood glucose levels by elevating glucokinase activity and production of glycogen in the liver.

KEYWORDS: Rice bran; phenolic acid; ferulic acid; hypoglycemic effect

INTRODUCTION

Noninsulin-dependent diabetes mellitus (NIDDM) is characterized by impaired secretion and action of insulin (*1*). Insulin secretion is abnormal in first-degree relatives of patients with NIDDM (*2*, *3*). This implies that defective insulin secretion is present at an early age and plays a primary role in the pathogenesis of NIDDM (*4*).

Insulin regulates glucose homeostasis by suppressing hepatic glucose production and stimulating peripheral glucose uptake (*2*). The synthesis of liver glycogen, the storage form of glucose, also plays a role in maintaining glucose homeostasis. Many studies have demonstrated that impaired insulin sensitivity or hyperglycemic hyperinsulinemia affects glucose storage in liver and muscle (*5*, *6*). Krssak et al. (*7*) monitored hepatic glycogen metabolism and endogenous glucose production after meal ingestion and showed that type 2 diabetic patients had low net rates of glycogen synthesis and high postprandial rates of glucose production. *p*-Methoxycinnamic acid and a phenolic cinnamic acid derivative present in plants increase glucose uptake and glycogen synthesis in hepatocytes (*8*, *9*) and affect the activities of hepatic glucose-regulating enzymes, resulting in improved glucose utilization in diabetes mellitus patients (*10*). Another study showed that phenolic compounds reduce glucose absorption from the small intestine by inhibiting α-glucosidase activity (*11*).

Most epidemiological studies have shown that whole grain is protective against diabetes, obesity, cancer, and cardiovascular disease (*12*–*14*). There are many potential mechanisms responsible for this protective effect because whole grains are rich in nutrients and phytochemicals (*15*). Rice bran in particular has many types of phenolic acids and biological activities. Dietary rice bran improves pancreatic insulin production and decreases blood glucose levels (*16*). Recent clinical studies on the treatment of NIDDM have focused on the potential use of plant constituents with insulin stimulatory abilities or on alleviation of insulin resistance. Nomura et al. (*17*) investigated the stimulatory effect of ferulic acid (FA), a type of phenolic acid, on insulin secretion in vitro and showed that exposure of RIN-5F cells to FA resulted in moderate stimulation of insulin activity. Several authors have reported that phenolic acids, including FA, have beneficial effects on diabetes (*16*, *17*). However, the effects of the phenolic and ferulic acids in rice bran on glucose metabolism in diabetes have received little attention.

We used C57BL/KsJ-*db/db* mice as NIDDM models to investigate the antidiabetic effects of FA and a phenolic acid fraction extracted from defatted rice bran.

MATERIALS AND METHODS

Materials. Folin-Ciocalteu reagent, catechin, FA, *p*-coumaric acid (PC), *m*-hydroxybenzoic acid, benzoic acid, and sinapic acid were

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Table 1. Composition of Experimental Diets (g/kg)

ingredient	modified diet
corn starch	549.5
sucrose	100.0
casein	200.0
corn oil	50.0
cellulose	50.0
AIN-76 MIN[a]	35.0
AIN-76 VIT[b]	10.0
DL-methionine	3.0
choline chloride	2.5
total	1000

[a] AIN-76 MIN: AIN-76 mineral mixture. [b] AIN-76 VIT: AIN-76 vitamin mixture.

purchased from Sigma Chemical Co. (St. Louis, MO, USA). Water and methanol for high performance liquid chromatography (HPLC) analysis were obtained from Fisher Scientific (Pittsburgh, PA). α-Glucosidase from baker's yeast (AGH, 5.7 U/mg) and the substrate, *p*-nitrophenyl α-D-glucopyranoside (PNPG), were purchased from Sigma Chemical Co.

Preparation and Analysis of a Phenolic Acid Fraction from Rice Bran. To extract the phenolic acid fraction (the ethyl acetate fraction, EAE), rice bran was defatted using *n*-hexane and subjected to extraction according to a method reported previously (*18*). In brief, 500g of defatted rice bran was suspended with 1 N NaOH at room temperature under a nitrogen atmosphere. The suspension was acidified to pH 1.5–2.5 by gradual addition of HCl and extracted three times with ethyl acetate. The ethyl acetate fractions were evaporated at 37 °C, and the residual material was redissolved in an 8% aqueous ethanol solution for animal supplementation or dissolved in methanol for analysis.

The composition of the EAE was analyzed using an HPLC (JASCO Co., Tokyo, Japan) equipped with a UV detector and a C_{18} column (μ-Bondapack, 3.9 × 300 mm internal diameter). The mobile phases were 0.05% phosphoric acid (A) and methanol (B), and the percentage of solvent B was increased in a gradient from 2 to 50% over 45 min during elution. Chromatography was performed at a flow rate of 0.5 mL/min, and the injection volume was 20 μL. Absorbance was determined at 280 nm.

Total Phenolic Content (TPC). The TPC of the extracts was determined according to the Folin-Ciocalteu method (*19*). EAE (200 μL) was mixed with 1.0 mL of 50% Folin-Ciocalteu reagent and 0.8 mL of 2% Na_2CO_3 and then allowed to stand for 30 min. Absorption was measured at 760 nm. Total phenolic levels were expressed as catechin equivalents.

α-Glucosidase (AGH) Inhibitory Activity. AGH inhibitory activity was determined by a partial modification of the procedure reported by Oki et al. (*20*), in which the enzyme solution was prepared at 0.2 U per assay volume of AGH in 0.1 M phosphate buffer (pH 7.0). For each assay, 20 μL of AGH inhibitor, 20 μL of enzyme solution, and 80 μL of PNPG were mixed and incubated at 37 °C for 30 min. The absorbance at 400 nm of *p*-nitrophenol released from PNPG was measured using a spectrophotometer. One unit of AGH activity was defined as the amount of AGH required to liberate one mole of *p*-nitrophenol from PNPG per minute.

Animal Experiment. Male eight-week-old type 2 diabetic mice (*db/db* mice) were purchased from Jackson Laboratory (Bar Harbor, Maine, US). Animals were initially fed a chow diet for 7 days for acclimation and randomly allocated to one of three groups. Animals were then fed a modified AIN-76 diet (American Institute of Nutrition, 1977) (**Table 1**), and two groups received oral administration of EAE (0.2 g/kg) or FA (0.05 g/kg; Sigma). All mice were housed individually in cages in a room with controlled temperature (24 °C) and lighting (light and dark alternating every 12 h). At the end of the experimental period, the mice were sacrificed under ether anesthesia after a 12 h fast. Blood was collected from the ophthalmic vein into EDTA-treated tubes, centrifuged at 1500*g* for 20 min at 4 °C, and stored at −70 °C. The livers and pancreases were excised, rinsed, weighed, frozen in liquid nitrogen, and stored at −70 °C.

Blood Analysis. Blood glucose concentrations were measured using a glucose analyzer and the glucose oxidase method (glucose test strip, Precision, Abbott Laboratory., Illinois). Plasma insulin levels were measured using an ELISA kit (Shibayagi Co., Shibukawa, Japan) and murine insulin standards. Concentrations of plasma total cholesterol and high density lipoprotein (HDL) cholesterol were measured using commercial kits (Shinyang Chemical Co., Seoul, Korea). HDL cholesterol was measured after precipitation of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) by phosphotungstic acid and magnesium chloride. LDL cholesterol concentrations were calculated according to a published equation (*21*).

Table 2. Phenolic Acid Content of the Phenolic Acid Fraction[a]

	phenolic acid fraction
m-hydroxybenzoic acid	12.22 ± 0.00
benzoic acid	35.96 ± 0.00
p-coumaric acid	78.98 ± 0.01
ferulic acid	303.42 ± 0.01
sinapic acid	96.33 ± 0.04
total	526.91

[a] mg per 100 g. Values are expressed as the mean ± SE.

Determination of Hepatic Glycogen Content. Glycogen content was determined by the modified method of Seifter et al. (*22*). Liver tissue was homogenized in five volumes of ice-cold 30% KOH and then incubated in a boiling water bath (100 °C) for 30 min. The glycogen was precipitated with ethanol and centrifuged at 3000*g* for 20 min at room temperature. The supernatant was removed, and the precipitate was dissolved in distilled water before addition of an anthrone reagent, which was allowed to react for a few minutes. Glycogen concentrations were measured at 620 nm using a spectrophotometer.

Determination of Hepatic Glucokinase Activity. Glucokinase activity was determined using a modification of the method of Davidson and Arion (*23*). Liver samples were homogenized in nine volumes of a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM mercaptoethanol, and 1 mM EDTA. Homogenates were centrifuged at 100000*g* for 1 h; the postmicrosomal supernatant was used for estimation of glucokinase activity. Glucokinase activity was estimated as the difference between glucose phosphorylation capacities at glucose concentrations of 100 and 0.5 mM, and hexokinase activity was estimated as glucose phosphorylating capacity at a concentration of 0.5 mM glucose. Glucokinase activity was estimated as the difference between glucose phosphorylation capacities at glucose concentrations of 100 and 0.5 mM. Protein content was determined according to the modified method of Lowry et al. (*24*) using bovine serum albumin as the standard.

Statistical Analysis. The data were expressed as the mean ± SE and analyzed using Duncan's multiple range test followed by estimation of least significant differences using the SAS program (Version 5.1). The relationships between variables were examined by Pearson correlation analysis. All statistical tests were evaluated at the 5% significance level.

RESULTS

Phenolic Acid and TPC Contents of the Phenolic Acid Fraction of Rice Bran. The yield of EAE from defatted rice bran was 0.7%. The yield of total phenolic acids was 41.65 mg of catechin equivalents per 100 g of defatted rice bran (data not shown). **Table 2** shows that the EAE contained a large quantity of phenolic acids (526 mg%), of which FA was the most abundant. The contents of FA, PC, sinapic acid, *m*-hydroxybenzoic acid, and benzoic acid in the phenolic acid fraction were 303, 78, 96, 12, and 35 mg %, respectively.

Inhibitory Activity of AGH. A spectroscopic method was used to screen EAE and phenolic acids (FA, PC, *m*-hydroxybenzoic acid, sinapic acid) for in vitro AGH inhibition. EAE and phenolic acids (FA, PC) strongly inhibited the activity of AGH from baker's yeast (**Table 3**). However, *m*-hydroxybenzoic acid and sinapic acid did not inhibit AGH activity (data

Table 3. Inhibition of α-Glucosidase Activity in Vitro[a]

	IC_{50}[b] (mg/mL)
EAE	0.22
FA	0.8
PC	0.66

[a] α-Glucosidase from baker's yeast. EAE, FA, and PC: phenolic acid fraction of rice bran, ferulic acid, and *p*-coumaric acid, respectively. [b] IC_{50} value is defined as the concentration of α-glucosidse inhibitor to inhibit 50% of its activity under the assayed conditions.

Table 4. Food Intake, Body Weight, and Organ Weights of Diabetic Mice Fed Experimental Diets for 17 Days[a]

	control	EAE	FA
food intake (g/day)	7.23 ± 0.34^{NS}	7.57 ± 0.10	6.69 ± 0.37
body weight before (g)	36.85 ± 0.69^{NS}	34.77 ± 1.20	37.02 ± 0.58
body weight after (g)	37.26 ± 1.50^{NS}	36.63 ± 1.25	35.30 ± 1.00
liver weight (g/100 g)	5.21 ± 0.18^{NS}	5.23 ± 0.18	5.04 ± 0.17
kidney weight (g/100 g)	1.29 ± 0.09^{NS}	1.13 ± 0.05	1.08 ± 0.07

[a] Mean values for all determinations based on $n = 8$. NS, not significant.

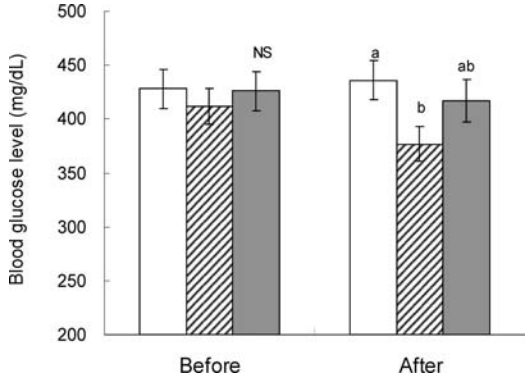

Figure 1. Blood glucose levels of diabetic mice before and after 17 days of administration of the experimental diets: open bars, control (diabetic control); hatched bars, EAE (diabetic plus 0.2 g/kg/day EAE); solid bars, FA (diabetic plus 0.05 g/kg/day FA). Means with different superscript letters are significantly different ($p < 0.05$, Duncan's multiple range test).

not shown). The AGH inhibitory activity of EAE, FA, and PC was concentration dependent. EAE had the strongest inhibitory activity, and the inhibitory activity of PC was stronger than that of FA.

Body Weight, Organ Weight, And Food Intake. The effects of EAE and FA on body weight, organ weight, and food intake are shown in **Table 4**. There were no significant differences in food intake or final body weight among the experimental groups. The hepatic weights of the FA group were slightly low, but not significantly so. The kidney weights of the EAE and FA groups were lower than those of the control group, but the difference was not significant.

Blood Glucose and Plasma Insulin Levels. Initial glucose levels in *db/db* mice ranged from 406.4 ± 112.46 mg/dL to 416.71 ± 51.32 mg/dL (**Figure 1**). After treatment with EAE and FA for 17 days, blood glucose levels were lower than those of the control group. The mean blood glucose levels of the EAE, FA, and control groups were 376.6 ± 16.08 mg/dL, 416.8 ± 19.54 mg/dL, and 436.0 ± 18.11 mg/dL, respectively. Administration of EAE decreased blood glucose levels by 13.6% relative to those of the control ($p < 0.05$).

Plasma insulin levels were significantly higher in the EAE and FA groups than in the control group (**Figure 2**). There was a negative correlation between blood glucose and plasma insulin levels ($r^2 = -0.2101$, $p < 0.05$) (**Figure 4**).

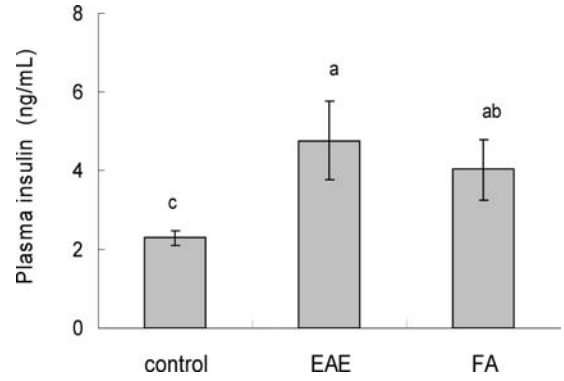

Figure 2. Plasma insulin levels in diabetic mice. All mice were fasted for 12 h before blood collection. Data are expressed as the mean ± SE ($n = 8$). The data showed significant differences at the level of $p < 0.05$.

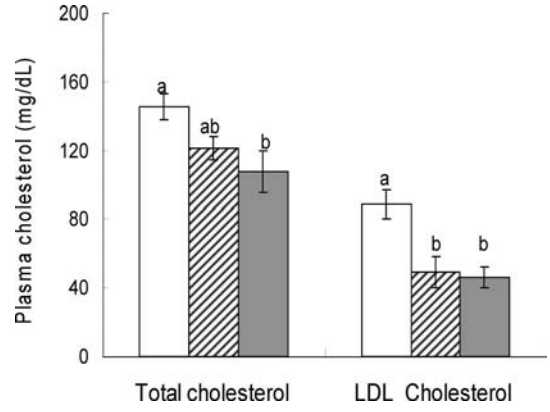

Figure 3. Change in concentrations of plasma total cholesterol and LDL cholesterol: open bars, control (diabetic control); hatched bars, EAE (diabetic plus 0.2 g/kg/day EAE); solid bars, FA (diabetic plus 0.05 g/kg/day FA). Means with different superscript letters are significantly different ($p < 0.05$, Duncan's multiple range test).

Hepatic Glucokinase Activity and Glycogen Concentration. We investigated whether EAE and FA affected the glucokinase activities and liver glycogen contents of *db/db* mice. As shown in **Table 5**, administration of EAE increased the activity of glucokinase by 46% relative to that of the control ($p < 0.05$). Administration of FA increased the activity of glucokinase slightly compared to that of the control group, but the difference was not significant. The hepatic glycogen content was significantly elevated ($p < 0.05$) in the EAE and FA groups compared to the control group. The liver glycogen contents of the EAE and FA groups were 27.12 ± 1.88 mg/g and 22.88 ± 1.01 mg/g, respectively. This result is similar to that of Robert et al. (*25*), who showed that hepatic glycogen production rates in NIDDM and hypoinsulinemic diabetic rat hepatocytes were 70 and 66% less than those of normal rat hepatocytes, respectively. There was a negative correlation between blood glucose and hepatic glycogen content ($r^2 = -0.1901$, $p < 0.05$), and glucokinase activity and plasma insulin level were positively correlated ($r^2 = 0.2983$, $p < 0.05$). When blood glucose concentrations exceed the threshold level, glucokinase begins to phosphorylate glucose to glucose-6-phosphate for glycogen synthesis (*26*). Therefore, elevated glucokinase activity (46%) may have played an important role in reducing hyperglycemia in this study.

Determination of Plasma Cholesterol Content. The effects of EAE and FA on the plasma cholesterol levels of diabetic mice are shown in **Figure 3**. The plasma total cholesterol level of the FA group was significantly lower than that of the control

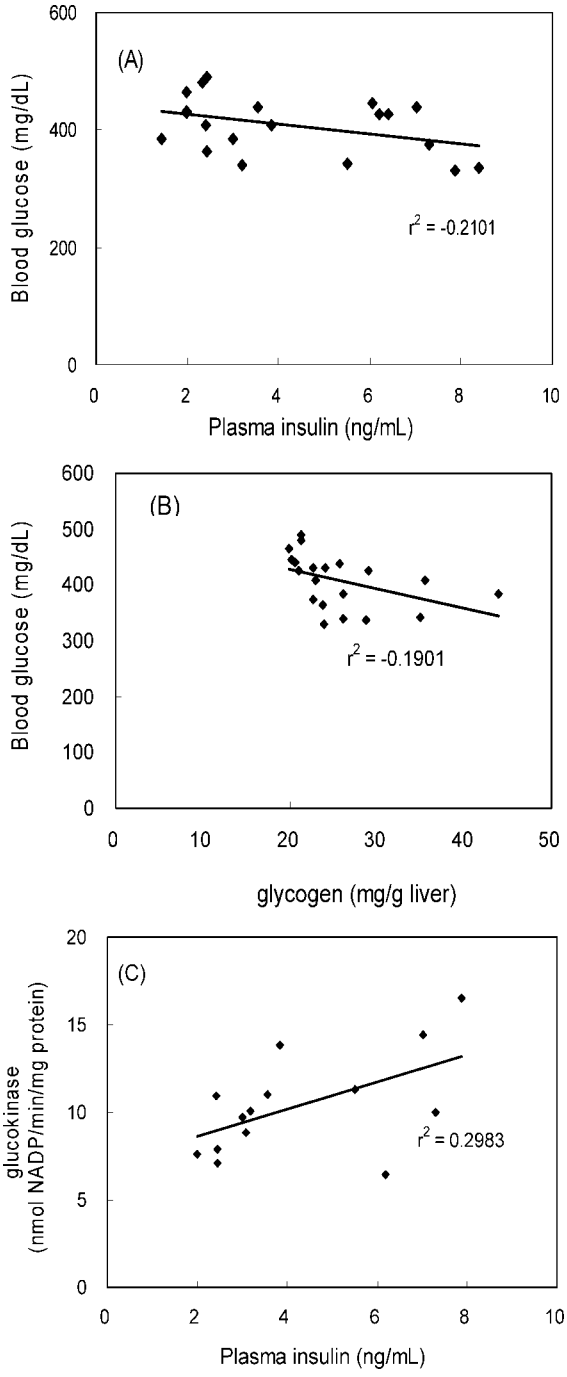

Figure 4. Correlations between concentrations of plasma insulin and blood glucose (**A**), concentrations of hepatic glycogen and blood glucose (**B**), and concentrations of plasma insulin and activity of hepatic glucokinase (**C**) in diabetic mice. Correlations were calculated using Pearson's correlation coefficients ($p < 0.05$).

group ($p < 0.05$). The LDL cholesterol concentrations of the EAE and FA groups were significantly lower than those of the control group.

DISCUSSION

C57BL/KsJ-*db/db* mice develop obesity, insulin resistance, hyperglycemia, and leptin resistance. As early as six weeks after birth, the *db/db* mouse already shows marked hyperglycemia and insulin resistance (*27*). These animals also have basal hypercholesterolemia when fed regular laboratory chow (*28*).

Table 5. Activity of Hepatic Glucokinase and Concentration of Hepatic Glycogen[a]

	control	EAE	FA
glycogen[b]	19.23 ± 2.09b*	27.12 ± 1.88a*	22.88 ± 1.01ab*
glucokinase[c]	8.84 ± 0.80b*	12.97 ± 1.46a*	9.64 ± 0.44ab*

[a] Data are expressed as the mean ± SE. Different letters in the same column are significantly different (*$p < 0.05$, Duncan's test). [b] Glycogen (mg/g of liver). [c] Glucokinase (nmol of NADP reduced/min/mg of protein).

Previous studies have shown that flavonoids and phenolic acids have anti-α-glucosidase and hypoglycemic properties in diabetics (*14,29*). For example, FA improves pancreatic insulin production in vitro and increases insulin receptor activity, which may explain how it modulates glucose metabolism in diabetics (*16, 30*).

We examined the hypoglycemic effects of phenolic acids in rice bran on glucose storage and utilization in diabetes, as there is little information on this aspect. We measured the effects of EAE and FA on in vitro AGH activity and in vivo hepatic glucokinase activity and glycogen storage. To measure the inhibitory activities of EAE and FA on glucose absorption from the small intestine, we used α-glucosidase from baker's yeast as enzyme and PNPG as substrate. The inhibitory activity of AGH was found in EAE, FA and PC, which is similar to a previous study that reported that *trans*-cinnamic acid derivatives inhibit AGH activity (*11*). EAE extracted from rice bran has considerable amounts of *trans*-cinnamic acid derivates, FA, and *p*-coumaric acids, which are hydroxycinnamic acids. EAE showed strong activity compared to the activity of each single compound, which may be due to a synergistic effect of phenolic acid in EAE.

Our study investigated whether EAE and FA affect regulation of glucose metabolism in postprandial *db/db* mice. After 17 days' administration of EAE and FA, blood glucose levels were significantly ($p < 0.05$) decreased in *db/db* mice. Plasma insulin levels were elevated in the EAE and FA groups. Despite hyperinsulinemia, the hepatic glucokinase activities and glycogen contents of the EAE and FA groups were elevated compared to those of the control group ($p < 0.05$). These results are similar to those reported by Aoki et al. (*31*). Plasma insulin level and glucokinase activity were positively correlated ($r^2 = 0.2983$, $p < 0.05$), and blood glucose level and hepatic glycogen content were negatively correlated ($r^2 = -0.1901$, $p < 0.05$).

Hepatic glucokinase is considered an important regulator of blood glucose levels. Glucokinase (hexokinase IV) is expressed predominantly in the liver and the β-cells, and glucokinase gene transcription is stimulated by insulin. This enzyme differs from the other mammalian hexokinase because of its low affinity for glucose (6–11 mM). The low affinity of glucokinase for glucose ensures that the rate of glucose phosphorylation is directly proportional to blood glucose levels. Therefore, the kinetic properties of glucokinase may afford it a key role in regulating and integrating glucose metabolism in the liver (*32, 33*). EAE and FA improved glucose regulation mainly via restoration of hepatic glucokinase and glycogen storage activity, resulting in reduced blood glucose levels. These results suggest that EAE and FA may increase insulin action and the utilization of dietary glucose in the liver.

Diabetes increases oxidative stress and levels of plasma total cholesterol and LDL cholesterol; these increased cholesterol levels are caused by diabetic dyslipidemia (*34*). The EAE and FA groups had significantly lower levels of plasma total cholesterol and LDL cholesterol compared with the control group. These results suggest that EAE and FA supplements may reduce the risk of diabetic hypercholesterolemia.

The phenolic acid fraction of rice bran appears to improve the utilization of plasma glucose by elevating glycogen production and activating hepatic glucokinase activity.

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