

The Hypoglycemic Effects of Hesperidin and Naringin Are Partly Mediated by Hepatic Glucose-Regulating Enzymes in C57BL/KsJ-db/db Mice¹

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ABSTRACT Dietary antioxidant compounds such as bioflavonoids may offer some protection against the early stage of diabetes mellitus and the development of complications. We investigated the effect of citrus bioflavonoids on blood glucose level, hepatic glucose-regulating enzymes activities, hepatic glycogen concentration, and plasma insulin levels, and assessed the relations between plasma leptin and body weight, blood glucose, and plasma insulin. Male C57BL/KsJ-db/db mice (db/db mice, 5 wk old), an animal model for type 2 diabetes, were fed a nonpurified diet for 2 wk and then were fed an AIN-76 control diet or the control diet supplemented with hesperidin (0.2 g/kg diet) or naringin (0.2 g/kg diet). Hesperidin and naringin supplementation significantly reduced blood glucose compared with the control group. Hepatic glucokinase activity and glycogen concentration were both significantly elevated in the hesperidin- and the naringin-supplemented groups compared with the control group. Naringin also markedly lowered the activity of hepatic glucose-6-phosphatase and phosphoenolpyruvate carboxykinase compared with the control group. Plasma insulin, C-peptide, and leptin levels in the db/db mice from the 2 bioflavonoid-supplemented groups were significantly higher than those of the control group. Furthermore, plasma leptin was positively correlated with plasma insulin level ($r = 0.578$, $P < 0.01$) and body weight ($r = 0.541$, $P < 0.05$), and was inversely correlated with the blood glucose level ($r = -0.46$, $P < 0.05$). The current results suggest that hesperidin and naringin both play important roles in preventing the progression of hyperglycemia, partly by increasing hepatic glycolysis and glycogen concentration and/or by lowering hepatic gluconeogenesis. *J. Nutr.* 134: 2499–2503, 2004.

KEY WORDS: • hesperidin • naringin • glucose regulating enzymes • C57BL/KsJ-db/db mice

C57BL/KsJ-db/db mice (db/db mice) develop diabetes mellitus due to a failure to respond to leptin, resulting from a mutation in their receptor gene expressed in the hypothalamus, although *ob* gene expression and leptin secretion are markedly augmented in these mice, resulting in leptin resistance (1). As such, db/db mice, also characterized by obesity, infertility, hyperphagia, temporary hyperinsulinemia, and hyperglycemia (2,3), are considered a good model for the early stage of type 2 diabetes mellitus, because they exhibit both hepatic and peripheral insulin resistance (1). The insulin insensitivity and insulin deficiency in several other animal models of type 2 diabetes mellitus lead to a decrease in blood glucose utilization by the liver, the muscles, and the adipose tissue, and to an increase in hepatic glucose production (4). Accordingly, an excessive hepatic glucose output would seem to be an important factor in the onset of hyperglycemia in type 2 diabetes mellitus (5–7).

Recently there has been a growing interest in hypoglycemic agents from natural products, especially those derived from plants (8–10), because plant sources are usually considered to

be less toxic, with fewer side effects than synthetic sources. Many traditional remedies for diabetes mellitus use plant sources, and over 200 pure phytochemicals are currently known to have hypoglycemic properties (11). Several bioflavonoids, ubiquitously present in plants, and common components of human diets have been reported to improve hyperglycemia in diabetes mellitus by affecting glucose transport (12,13), insulin-like properties (14), and insulin-receptor function (15). Hesperidin and naringin, both citrus bioflavonoids, exhibit biological and pharmacological properties, such as anti-inflammatory, anticarcinogenic, lipid-lowering, and antioxidant activities (16,17). However, very little is known about the biochemical mechanism of the hypoglycemic effect of these citrus bioflavonoids.

Accordingly, the present study was performed to examine the role of hesperidin and naringin in regulating blood glucose, plasma insulin, and hepatic glycogen levels in db/db mice, type 2 diabetic mice, along with the activities of the hepatic glucose-regulating enzymes involved in glycolysis and gluconeogenesis.

MATERIALS AND METHODS

Animals and diets. Thirty male 5-wk-old (23 g), type 2 diabetic mice (db/db mice) were purchased from Jackson Laboratory. All mice were individually housed in stainless-steel cages in a room with controlled temperature (24°C) and lighting (alternating 12-h periods

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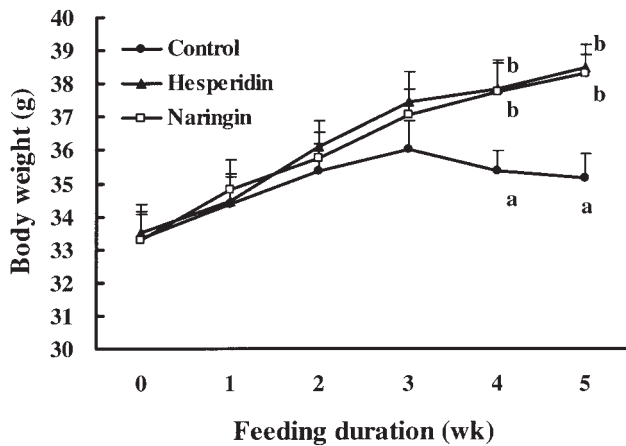


FIGURE 1 Body weight in C57BL/KsJ-db/db mice fed diets supplemented with hesperidin or naringin. Values are means \pm SEM, $n = 10$. Means without a common letter differ, $P < 0.05$.

of light and dark). Upon arrival, all the mice were fed a pelleted commercial diet (Samyang) for 2 wk, then they were randomly divided into 3 groups ($n = 10$) and were fed a standard laboratory diet (AIN-76) (18,19) with no supplement, a hesperidin supplement (0.2 g/kg diet, Sigma Chemical), or a naringin supplement (0.2 g/kg diet, Sigma Chemical) for 5 wk. Mice had free access to food and water. We measured food consumption and body weight every day and every week, respectively. At the end of the experimental period, the mice were anesthetized with ketamine after withholding food for 12 h, and blood samples were drawn from the inferior vena cava for the determination of plasma insulin, C-peptide, and leptin. Also, the livers were removed after collecting blood, rinsed with physiological saline solution, weighed, and immediately stored at -70°C . Mice were treated in accordance with the Kyungpook National University Guide for the Care and Use of Laboratory Animals.

Blood biomarkers. The blood glucose concentration was measured at 7, 8, 9, 10, 11, and 12 wk of age, after 0, 1, 2, 3, 4, and 5 wk of hesperidin or naringin supplementation, respectively. After with-

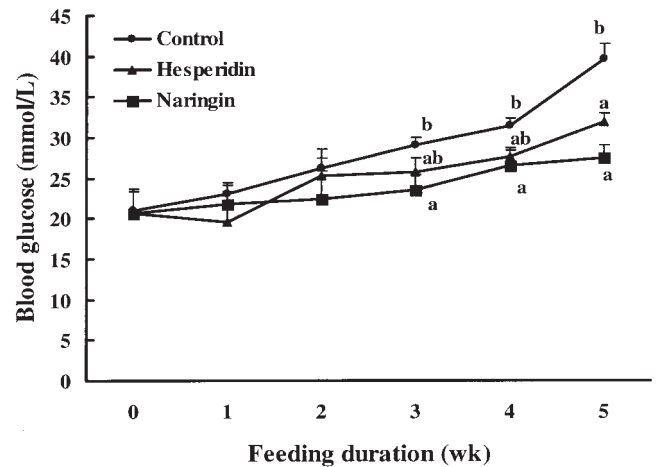


FIGURE 2 Blood glucose levels in C57BL/KsJ-db/db mice fed diets supplemented with hesperidin or naringin. Values are means \pm SEM, $n = 10$. Means without a common letter differ, $P < 0.05$.

holding food for 6 h, the blood glucose concentration was measured, with whole blood obtained from the tail veins, by using a glucose analyzer based on the glucose oxidase method (Glucocard test strip 127, Arkray). Plasma insulin (RIA kit, Diagnostic Systems Laboratories), C-peptide (C-peptide RIA kit, Diagnostic Systems Laboratories), and leptin (Mouse leptin RIA kit, Linco Research) levels were measured based on a radioimmunometric assay.

Hepatic glycogen assay. The glycogen concentration was determined as previously described by Seifter et al. (20), with modifications. Briefly, the liver tissue was homogenized in 5 volumes of an ice-cold 300 g/L KOH solution and was dissolved in a boiling water-bath (100°C) for 30 min. The glycogen was precipitated with ethanol, and then was pelleted, washed, and resolubilized in distilled water. The glycogen concentration was determined by treatment with an anthrone reagent [2 g anthrone per 1 L of 95% (v/v) H_2SO_4] and by measuring the absorbance at 620 nm.

Hepatic enzyme activities. Glucokinase activity was determined from liver samples homogenized in 9 volumes of a buffer containing

TABLE 1

The activities of hepatic glucokinase, glucose-6-phosphatase, and phosphoenolpyruvate carboxykinase in C57BL/KsJ-db/db mice fed diets containing hesperidin or naringin¹

	Control	Hesperidin	Naringin
	<i>nmol · min⁻¹ · mg protein⁻¹</i>		
Glucokinase	278.55 \pm 7.91 ^a	375.66 \pm 9.81 ^b	373.00 \pm 8.74 ^b
Phosphoenolpyruvate carboxykinase	118.32 \pm 3.93 ^b	104.17 \pm 8.60 ^{a,b}	95.93 \pm 5.33 ^a
Glucose-6-phosphatase	941.93 \pm 64.41 ^b	801.11 \pm 53.77 ^{a,b}	676.38 \pm 51.00 ^a

¹ Values are means \pm SEM, $n = 10$. Means in a row without a common letter differ, $P < 0.05$.

TABLE 2

Concentrations of plasma insulin, C-peptide, leptin, and hepatic glycogen in C57BL/KsJ-db/db mice fed diets supplemented with hesperidin or naringin¹

	Control	Hesperidin	Naringin
Insulin, pmol/L	202.10 \pm 12.62 ^a	327.66 \pm 47.11 ^b	301.35 \pm 21.53 ^b
C-peptide, pmol/L	199.80 \pm 2.35 ^a	299.70 \pm 2.11 ^c	233.10 \pm 2.35 ^b
Leptin, $\mu\text{g/L}$	49.10 \pm 3.16 ^a	61.34 \pm 2.96 ^b	58.58 \pm 3.02 ^b
Glycogen, mg/g liver	63.37 \pm 0.55 ^a	65.25 \pm 0.62 ^b	65.65 \pm 0.18 ^b

¹ Values are means \pm SEM, $n = 10$. Means in a row without a common letter differ, $P < 0.05$.

50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L KCl, 10 mmol/L mercaptoethanol, and 1 mmol/L EDTA. Homogenates were centrifuged at $100,000 \times g$ for 1 h; the postmicrosomal supernatant was used for the spectrophotometric continuous assay as described by Davidson and Arion (21), with a slight modification, whereby the formation of glucose-6-phosphate from glucose at 27°C was coupled to its oxidation by glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide (NAD).

Glucose-6-phosphatase activity was determined in the hepatic microsome by using a spectrophotometric assay according to the method of Alegre et al. (22), with a slight modification; the reaction mixture contained 100 mmol/L sodium Hepes (pH 6.5); 26.5 mmol/L glucose-6-phosphate and 1.8 mmol/L EDTA, both previously adjusted to pH 6.5; 2 mmol/L NADP⁺; 0.6 kIU/L mutarotase; and 6 kIU/L glucose dehydrogenase.

Phosphoenolpyruvate carboxykinase (PEPCK) activity was measured by using the spectrophotometric assay developed by Bentle and Lardy (23), with a slight modification. A 1-mL final volume of the purified hepatic cytosolic enzyme pipetted with the reaction mixture, pH 7.0, contained 50 mmol/L sodium Hepes, 1 mmol/L IDP, 1 mmol/L MnCl₂, 1 mmol/L dithiothreitol, 0.25 mmol/L nicotinamide adenine dinucleotide, reduced form (NADH), 2 mmol/L phosphoenolpyruvate, 50 mmol/L NaHCO₃, and 7.2 U of malic dehydrogenase. The enzyme activity was measured at 25°C, based on a decrease in the absorbance at 340 nm.

Pancreas histology and immunohistochemistry. Briefly, the pancreas was rapidly removed after decapitation and was fixed in 10% (v:v) neutral buffered formalin, was processed routinely, and was embedded in paraffin wax. Paraffin sections were cut at 4- μ m thickness and were deparaffinized in xylene twice for 5 min and then were rehydrated with the graded ethanol. The sections were stained with hematoxylin and eosin, and, for immunohistochemistry, rehydrated sections were treated with 3% (v:v) H₂O₂ in methanol for 30 min to block endogenous peroxidase, were washed with 0.01 mol/L phosphate buffer for 10 min, and then were immunostained with the primary antibody, monoclonal mouse anti-insulin. The antigen-antibody complex was visualized by an avidin-biotin peroxidase complex solution by using an ABC kit (Vector Laboratories) with 3,3'-diamino benzidine (Zymed Laboratories). For the negative control, the primary antibody was replaced by phosphate-buffered saline solution.

Statistical analysis. All data are presented as means \pm SEM. Significant differences among the groups were determined by one-way ANOVA with SPSS 11.0 for Windows. Duncan's multiple-range test was performed when the *F*-test was significant ($P < 0.05$). Pearson correlation coefficients were calculated to examine the association of the leptin levels with body weight, plasma insulin, and blood glucose.

RESULTS

Body-weight gain, relative organ weight, and food intake.

The body weights of the two citrus bioflavonoid-supplemented groups were significantly higher than that of the control group at wk 4 and 5 of the experimental period (Fig. 1). Food intakes and relative organ weights did not differ among the groups (data not shown).

Blood glucose levels. All the db/db mice were diabetic when the experiment began, as indicated by their blood glucose levels (≥ 20.66 mmol/L). The blood glucose level of the naringin-supplemented group was significantly lower than that of the control group at wk 3, 4, and 5 of the experimental period, whereas the blood glucose level of the hesperidin-supplemented group was significantly lower than that of the control group only at wk 5 (Fig. 2).

Hepatic enzyme activities and glycogen concentration.

The hesperidin and naringin supplements significantly elevated hepatic glucokinase activity when compared with the control group by about 35% (Table 1). In contrast, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities were markedly lower in the db/db mice supplemented

with naringin compared with the control group, by 28.19% and 18.92%, respectively (Table 1). The hepatic glycogen concentration was significantly higher in the both hesperidin- and naringin-supplemented groups compared with the control group (Table 2).

Plasma insulin, C-peptide, and leptin levels. The plasma insulin, C-peptide, and leptin levels of the hesperidin- and naringin-supplemented groups were significantly higher than those of the control group (Table 2). The plasma leptin level was positively correlated with body weight ($r = 0.541$, $P < 0.05$) and plasma insulin level ($r = 0.578$, $P < 0.01$) (Fig. 3). In contrast, plasma leptin and blood glucose levels were inversely correlated ($r = -0.46$, $P < 0.05$) (Fig. 3).

Pancreas histology and immunohistochemistry. The islets of Langerhan in db/db mice appeared to have normal architecture. Intact and specific insulin-positive cells were confined

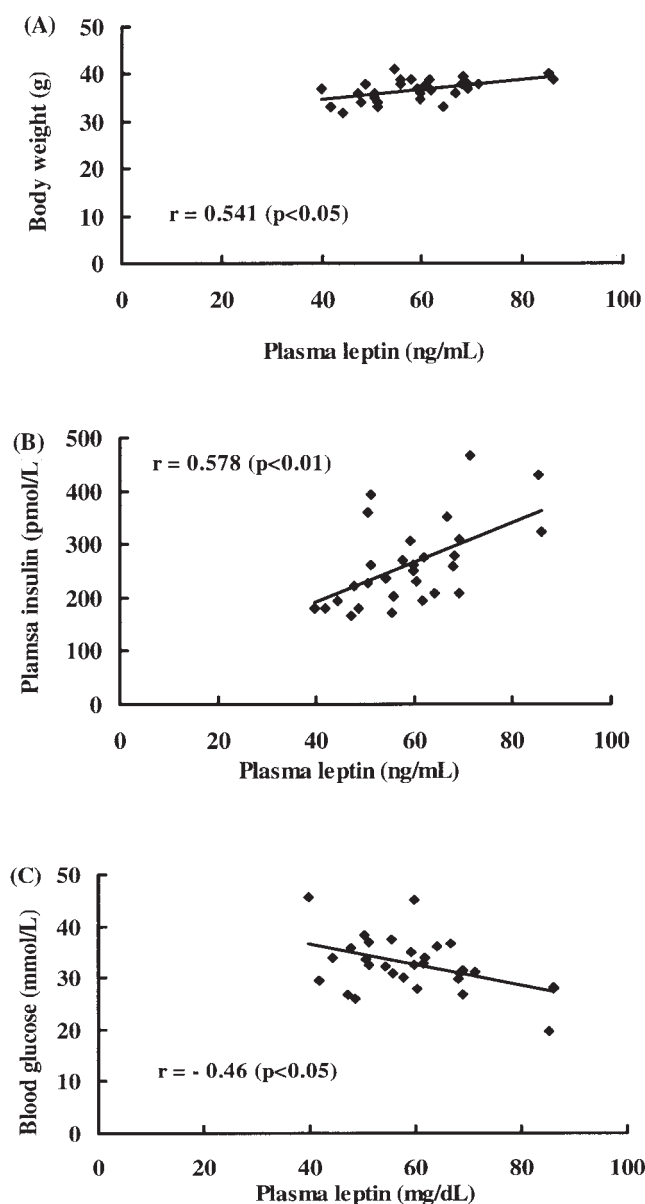
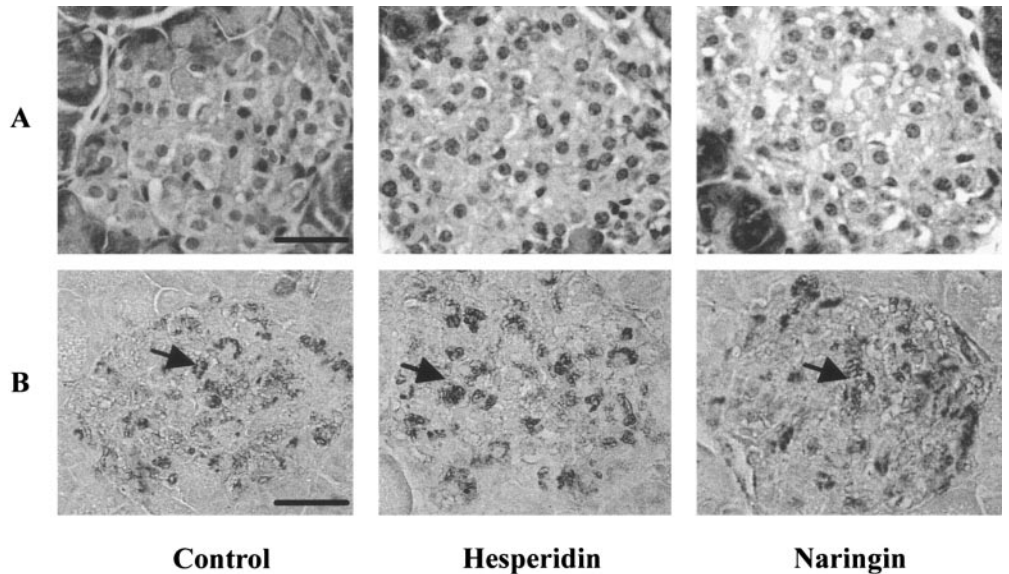


FIGURE 3 Correlations between plasma leptin level and body weight (A), plasma insulin level (B), and blood glucose level (C) in C57BL/KsJ-db/db mice fed diets supplemented with hesperidin or naringin.

FIGURE 4 Histological (A) and immunohistochemical (B) photomicrograph of pancreas in C57BL/KsJ-db/db mice fed diets supplemented with hesperidin or naringin. Arrows indicate insulin-producing cells located on central areas of pancreatic islets. Original magnification X132. Bar, 50 μ m.



to the pancreatic islet β cells, regardless of hesperidin or naringin supplementation (Fig. 4).

DISCUSSION

Hesperidin and naringin both significantly lowered the blood glucose level compared with the control group by 20 and 31%, respectively, after 5 wk of supplementation. The hypoglycemic effect of hesperidin and naringin seemed to be mediated by changes in the hepatic glucose regulating enzyme activities in the db/db mice. In general, increased hepatic glucose production, plus decreased hepatic glycogen synthesis and glycolysis, are the major symptoms in type 2 diabetes that result in hyperglycemia, and these would seem to be the consequence of the low glucokinase activity and high glucose-6-phosphatase and PEPCK activities in a diabetic state (5–7). Hepatic glucokinase is the most sensitive indicator of the glycolytic pathway in diabetes and its increase can increase the utilization of blood glucose for glycogen storage in the liver (24). In the current study, supplementation of hesperidin and naringin in db/db mice increased hepatic glucokinase. Also, hepatic glycogen reserves are important for whole-body glucose homeostasis and are markedly low in the diabetic state (25–27). In the current study, the hepatic glycogen concentration was significantly higher in the hesperidin- and naringin-supplemented groups compared with the control group. Naringin not only markedly elevated the hepatic glucokinase activity but also significantly lowered the hepatic glucose-6-phosphatase and PEPCK activities compared with the control group, which may explain why the glucose-lowering effect of naringin appeared earlier than that of hesperidin in this study.

Chronic insulin deficiency and insulin insensitivity are the major causes of the decreased hepatic glucose utilization and increased glucose production in several animal models of type 2 diabetes, e.g., db/db mice, because insulin decreases the hepatic glucose output by activating glycogen synthesis and glycolysis, and by inhibiting gluconeogenesis (4). Generally, in db/db mice, the plasma insulin level increases rapidly during the first few weeks of life, then declines successively after 8–10 wk of age, resulting in a drastic decrease in body weight at the time of death (1). In the present study, the levels of plasma insulin and C-peptide, indices of insulin secretion (28), in the db/db mice from the hesperidin- and naringin-supplemented

groups were significantly higher than those in the control group at 12 wk of age, at the end of the study. The plasma insulin levels in the db/db mice may have decreased after reaching a peak, while the supplementation of naringin and hesperidin prevented the decline in the plasma insulin levels. However, the plasma insulin level was not measured throughout the current study.

Previous studies (29–31) have shown that high blood glucose causes the deterioration of pancreatic β cells due to oxidative stress. Therefore, antioxidants can have beneficial effects on pancreatic β cells by neutralizing the oxidative stress. Normal β cells compensate for insulin resistance by increasing glucose-stimulated insulin secretion or β -cell mass (29). Astaxanthin, an antioxidant, has been found to preserve the ability of β cells to secrete insulin, although no significant difference was found in the β -cell mass between astaxanthin-treated and untreated db/db mice (32). In the current study, the higher plasma insulin levels in the hesperidin- and naringin-supplemented groups than in the control group may have been mediated via the stimulation of insulin secretion in the β cells, because intact and specific insulin-positive cells were confined to the pancreatic islet β cells, regardless of hesperidin or naringin supplementation. This may have been partly because β -cell destruction, such as islet atrophy and necrosis, is generally only exhibited at about 4 mo of age in db/db mice (33), while the current study was terminated when the mice were 3 mo old.

The plasma insulin and leptin levels are generally correlated, because insulin stimulates leptin synthesis and release through the regulation of glucose metabolism in the adipocytes (34,35). The present study also revealed a positive correlation between the plasma leptin and insulin levels ($r = 0.578$, $P < 0.01$). Although there was no difference in the adipose tissue weight among the groups, the plasma leptin level and body weight were positively correlated ($r = 0.541$, $P < 0.05$). Several previous studies have reported that type 2 diabetic patients have significantly lower leptin levels than nondiabetic subjects (36). Furthermore, it has been reported that insulin sensitivity is enhanced in mice that overexpress leptin (37), suggesting that low levels of leptin with type 2 diabetes may also directly increase insulin resistance, thereby worsening the condition. However, an inverse association between plasma

leptin and blood glucose was observed in the current study ($r = -0.46$, $P < 0.05$), and Moriya et al. (38) also reported an inverse relationship between plasma leptin levels and blood glucose in type 2 diabetic patients.

In conclusion, the present study suggests that supplementation with hesperidin or naringin improves hyperglycemia in type 2 diabetic db/db mice by, at least in part, increasing glucose utilization, which seemingly was mediated via elevated glycolysis and hepatic glycogen concentration resulting from the effect on glucokinase. In particular, naringin regulated gluconeogenesis by lowering the activities of glucose-6-phosphatase and PEPCK. In addition, the levels of plasma insulin, C-peptide, and leptin in the hesperidin- and naringin-supplemented groups were significantly higher than those in the control group. However, further studies are needed to elucidate the mode of action on enhanced insulin release by these bioflavonoids.

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