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Enzogenol improves diabetes-related metabolic change in C57BL/KsJ-*db*/*db* mice, a model of type 2 diabetes mellitus

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Keywords
Enzogenol; gluconeogenesis; glucose homeostasis; insulin resistance; type 2 diabetes

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Abstract

Objectives Dietary use of pine bark extract has been associated with reduced risk of inflammation and diabetes. In this study, we investigated the antidiabetic effects of enzogenol, proanthocyanidins-rich bioflavonoid extract derived from the pine bark of New Zealand Pinus radiata trees, using C57BL/KsJ-*db*/*db* mice.

Methods After 1-week acclimation period, the *db*/*db* mice were divided into vehicle-treated, Enzogenol-treated (12.5, 25 and 50 mg/kg; EZ) and positive control (tea polyphenol 50 mg/kg; TPP) groups.

Key findings The administration of EZ improved the glucose tolerance and lowered the glycosylated haemoglobin (HbA_{1C}), insulin and glucagon levels in blood. Interestingly, EZ and TPP treatments resulted in reduced hepatic free fatty acid, cholesterol and triglyceride levels in *db*/*db* mice. EZ and TPP treatments significantly elevated hepatic AMPK activity, and the expression of proteins related to glucose homeostasis and lipid metabolism, such as glucokinase, peroxisome proliferator-activated receptor (PPAR)α and long-chain acyl-CoA dehydrogenase protein level with a simultaneous reduction of glucose-6-phosphatase and phospho*enol*pyruvate carboxykinase protein expression. In addition, the EZ administration groups had an increased hepatic glycogen synthase expression in *db*/*db* mice.

Conclusions These results suggest that EZ may be beneficial in improving insulin resistance and hyperglycaemia in type 2 diabetic mice by enhancing the glucose and lipids metabolism.

Introduction

Maritime pine bark extract is one of the most potent natural antioxidant and anti-inflammatory properties attributed to the bioflavonoids composition of proanthocyanidins, monomeric flavonoids and phenolic acids, and its diverse pharmacological activity has been well documented.[1,2] Recently, it has been reported that dietary pine bark extract reduces the development of atherosclerotic lesions in male apolipoprotein E-deficient mice by lowering the serum cholesterol level, suggesting its anti-atherogenic effects.[3] Moreover, a randomized and double-blinded clinical study has shown that the treatment of pine bark extract improved both cognitive and cardiovascular functions in the group of older adults.[4] In the long-term study, the dietary supplementation with combined Enzogenol (EZ) and vitamin C was not associated with any adverse change in laboratory markers of renal and liver function, glycaemic control and haematology.[5]

A standardized bark extract that complies with the monograph of maritime pine bark extract is derived from *Pinus pinaster*, *Ait*. (Pycnogenol). About 65–75% of this extract are procyanidins consisting of catechin and epicatechin moieties of varying chain lengths.[6] Pycnogenol (PZ) is known to possess potent antioxidant activity. It does not only scavenge the free radicals, but also enhances the endogenous antioxidant systems.[7] These properties have led to their long-term use in treating inflammation,[8] diabetic retinopathy[9] and cardiovascular disease associated with type 2 diabetes.[10]

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by insulin resistance in the

peripheral tissues, as well as progressive β-cell dysfunction leading to hyperglycaemia.[11] Different risk factors or events can cause T2DM, such as obesity and age.[12] The functional disturbance of pancreatic β-cells causes hyperglycaemia by a decrease in glucose utilization in the liver, muscle and adipose tissue, and an increase in hepatic glucose production.[13] Liver plays an important role in the glucose homeostasis through glycolysis, glycogenesis and gluconeogenesis. Glucagon regulates blood glucose by affecting glucose metabolism, specifically by increasing gluconeogenesis and decreasing glycolysis *in vivo*. The net glucose uptake by the liver depends on the activity of glucokinase (GCK) and glucose-6-phosphatase (G-6-Pase).[14] Glucagon has been shown to increase G-6-Pase expression and activity, which is the last step of the pathway. Phospho*enol*pyruvate carboxykinase (PEPCK) catalyses the conversion of oxaloacetate into phospho*enol*pyruvate, which is the early and rate-limiting step in the pathway of hepatic gluconeogenesis by glucagon action. Also, glucagon inhibits glycogen synthesis by regulating glycogen synthase in the liver.[15] The activation of AMPK has also been shown to reduce expression levels of molecules involved in gluconeogenesis such as PEPCK and G-6-Pase in hepatocytes.[16] Also, AMPK suppresses the expression of lipogenesis-associated genes, such as fatty acid synthase, pyruvate kinase and ACC,[17–21] resulting in reduced malonyl CoA levels and increased fatty acid oxidation.[22] It was recently demonstrated that the activation of AMPK by resveratrol protected against lipid accumulation in the liver of diabetic mice.[23]

Enzogenol (EZ) is a complex mixture of plant phenolic compounds, including many different flavonoids and phenolic acid that occur naturally from the pine bark of New Zealand Pinus radiata trees.[24] Proanthocyanidins, the most abundant flavonoid component of pine bark extracts, are flavan-3-ols composed most commonly of 2–10 units of catechin and epicatechin connected by different inter-flavan linkages resulting in varying oligo- and polymeric structures (Figure 1).[2] In contrast to PZ, which contains a wide variety of procyanidins that range from the monomeric catechin and taxifolin to oligomers with seven or more flavonoid subunits,[25] the most abundant groups of phenolics in EZ are oligomeric proanthocyanidins.[26]

A number of experimental and clinical studies have shown the hypolipidemic, antioxidant and other beneficial effects of PZ in type 2 diabetes.[27,28] However, the antidiabetic effect of EZ is not well delineated. Here, we investigated the antidiabetic effect of EZ in *db/db* mice. Blood glucose concentration, insulin secretion, insulin resistance and its effect on the expression of the glucose metabolism regulating genes in the diabetic C57BL/KsJ-*db/db* mice were examined.

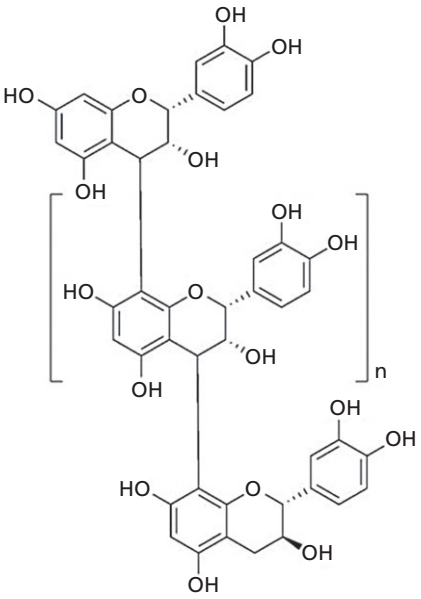

Figure 1 Structure of oligo- and poly-proanthocyanidins formed from catechin and epicatechin. Information for proanthocyanidins form of Enzogenol was provided from ENZO Nutraceuticals Ltd. The proanthocyanidins (often referred to as OPCs = oligomeric proanthocyanidins) are the most abundant group of phenolics in Enzogenol with more than 80% by weight.

Materials and Methods

Materials

EZ was kindly provided by ENZO Nutraceuticals Ltd. (Auckland, New Zealand). Components information of Enzogenol used in this study was published by Frevel laboratory.[2] Following Frevel *et al.*, EZ contained proanthocyanidins 84.3% (±3.7), taxifolin 1.47% (±0.14) and catechin 0.67% (±0.08). Tea polyphenol, positive control, was obtained from Amore Pacific Co. (Yongin-si, Gyeonggi-do, Korea); it contained 30% epigallocatechin gallate (EGCG). Protease and phosphatase inhibitor cocktails were purchased from Roche (Mannheim, Germany). Immunoblotting was performed using the following antibodies: anti-PEPCK (ab70358) and GCK (ab37796) from Abcam (Cambridge, UK); β-tubulin (sc-5274), G-6-Pase (sc-134714) and AMPK (sc-25792) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); glycogen synthase (#3893), p-AMPK (#2531S), anti-rabbit (#7074) and anti-mouse (#7076) IgG horseradish peroxidase (HRP)-linked antibody from Cell Signaling Technology, Inc. (Beverly, MA, USA); long-chain acyl-CoA dehydrogenase (LCAD) (17526-1-AP) from ProteinTech Group, Inc. (Chicago, IL, USA); and PPAR-α (PA1-822A) from Thermo Scientific (Rockford, IL, USA).

In-vivo experimental design

Six-week-old male C57BL/KsJ *db/db* mice and non-diabetic mice (C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals were individually housed in stainless steel cages, and adapted to 22 ~ 28°C and to the light–dark cycle (12 h–12 h). The 6-week-old *db/db* mice and non-diabetic mice were fed a pelletized commercial chow diet (Cargill Agri Purina Inc., Seongnam, Gyeonggi, Korea) for a period of 1 week after arrival and then randomly divided into six groups: normal control, diabetes control, positive control (tea polyphenol containing 70% catechins, 50 mg/kg; TPP) and EZ (12.5, 25 and 50 mg/kg) groups ($n = 8$), respectively. TPP and EZ were dissolved in distilled water and administered orally once a day for 6 weeks. The mice had free access to food and distilled water. Food and water consumption and weight gain were measured three times a week.

All of the experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees (Approval No. KHP-2010-04-21).

Blood and tissue sampling

At the end of treatment, animals were fasted overnight. Blood samples were drawn from the tail vein and the inferior vena cava to determine serum biomarkers. After collecting blood samples, the liver was immediately removed and stored at −80°C for subsequent determination of lipid parameters and protein levels.

Blood glucose and HbA_{1C} concentrations

Blood glucose concentration was measured using glucometer (GlucoDr; All Medicus Co., Anyang-si, Gyeonggi-do, Korea). The blood HbA_{1C} concentration was measured using EASY A1C™ (Infopia Co., Anyang-si, Gyeonggi-do, Korea).

Oral glucose tolerance test

At the end of EZ treatment, oral glucose tolerance test (OGTT) was performed after an overnight fast. The animals were fed glucose (3.0 g/kg of body weight) solution by oral administration. Blood samples were collected from the tail vein before, and 30, 60, 90 and 120 min after, glucose administration. Blood glucose levels were measured using GlucoDr (All Medicus Co.).

Measurements of serum insulin and glucagon levels

Blood samples were collected from the interior vena cava, drawn into Vacutainer (Becton Dickinson & Co., Rutherford, NJ, USA) and centrifuged (890*g* for 15 min at 4°C) to separate the serum from blood cells. Insulin and glucagon levels in serum were measured by enzyme-linked immunoassays. Mouse insulin ELISA kit was purchased from Shibayagi (Shibukawa, Japan), and mouse glucagon ELISA kit was purchased from USCN LIFE (Wuhan, China).

Western blotting for hepatic gluconeogenesis and beta-oxidation

Lysates were prepared using lysis buffer (20 mM Tris-HCl (pH 7.4), 0.32 mM sucrose, protease inhibitor, 1 mM PMSF, 0.5 M EDTA (pH 8.0), 1 mM NaF and 1 mM Na_3VO_4). One hundred micrograms of protein per lane was separated by SDS-polyacrylamide gel (8 or 10%) electrophoresis. Proteins were transferred onto Polyvinylidene fluoride membranes in transfer buffer (25 mM Tris-HCl (pH 7.4), 192 mM glycine and 20% v/v methanol). The transferred membranes were incubated for 2 h in blocking solution (5% dried milk in Tris-buffered saline containing 0.1% Tween-20) at room temperature. Blots were incubated with the appropriate primary antibodies at a dilution of 1 : 1000, and then further incubated with HRP-conjugated secondary antibody at a dilution of 1 : 5000. Bound antibodies were detected using enhanced chemiluminescence plus kits (Amersham International, Little Chalfont, UK). The PEPCK, GCK, G-6-Pase, AMPK, p-AMPK, glycogen synthase, LCAD and PPAR-α bands were detected with rabbit polyclonal anti-antibody (1 : 1000), respectively. The β-tubulin band was detected with mouse monoclonal anti-β tubulin antibody (1 : 1000).

Measurements of hepatic lipid levels

Hepatic lipids were extracted by Folch method.[29] The levels of triglyceride (TG), total cholesterol (TC) and HDL cholesterol (HDL-C) were determined by enzymatic method (Asan Pharm. Co. Ltd, Whaseong-si, Gyeonggi-di, Korea), and the free fatty acid (FFA) was determined using Labassay NEFA (Wako Chemicals, Richmond, VA, USA).

Statistic analysis

The results were presented as mean ± SE. Statistically significant differences between the groups were determined by statistical package for social sciences (SPSS; Chicago, IL, USA) using one-way analysis of variance. Multiple comparisons were performed with Tukey's test as described. The data were considered significantly different at $P < 0.05 \sim 0.001$.

Results

Body weight, food and water intake

The increase of body weight during the 6-week experimental period in the diabetic control group was significantly higher as compared with that of control group, as expected, although the average body weight in the *db/db* mice group was higher than that in the non-diabetic control group (Table 1). EZ medium- and high-dose or TPP-treated group showed decreased body weight gain compared with the diabetic control group, although they were not statistically significant. During the experimental period, the food intake in *db/db* mice group was approximately 2.2-fold higher than that in non-diabetic group. EZ medium and high-dose or TPP administration did not exhibit a significant change of cumulative food intake. The food efficiency ratios of the EZ- (medium and high-dose) or TPP-treated groups were lower than that of the diabetic control group; however, EZ low-dose-treated group was highest among the groups. The water intake in diabetic control group was significantly higher than that in the non-diabetic control and sample treated group. EZ treatment reduced water intake in *db/db* mice.

Fasting blood glucose, oral glucose tolerance test and the area under curve of oral glucose tolerance test

To assess the effect of EZ in glucose level, we treated *db/db* mice with various doses of EZ and examined the blood glucose levels. The administration of EZ or TPP tended to lower the blood glucose level compared with the diabetic control group during the experimental period in a dose-dependent manner (Figure 2a, Table 2). The treatment of EZ or TPP decreased blood glucose level by 10.1% (EZ, 12.5 mg/kg), 19.6% (EZ, 25 mg/kg) and 40.6% (EZ, 50 mg/kg), respectively (Figure 2a, Table 2). In addition, 50 mg/kg EZ treatment (40.6%) reduced blood glucose level more effectively than TPP treatment (31.2%). To further determine the effect of EZ on blood glucose level, we performed OGTT, which measures the ability to clear the circulating blood glucose (Figure 2b, Table 3). After glucose administration, the rate of blood glucose removal change in non-diabetic group significantly higher than all experimental groups showed only 10% increased blood glucose level. In contrast, a rapid increase of blood glucose level was observed as early as 30 min after glucose administration and remained elevated for at least 120 min in *db/db* mice, suggesting that diabetic control mice have declined glucose-handling ability. Interestingly, EZ treatment caused a rapid removal of blood glucose compared with diabetic control group ($P < 0.05$). As shown in Figure 2c, the glucose incremental area under the curve was significantly induced in diabetic control than that in non-diabetic control group. But EZ medium- and high or TPP-treated group significantly reduced compared with diabetic control group.

$HbA1_C$, serum insulin and glucagon levels

To further understand the antidiabetic effect of EZ, we examined the HbA1c, insulin and glucagon levels in blood. Blood HbA1c concentrations of the diabetic control group were 2.6-fold ($P < 0.001$) higher than that of non-diabetic group. Medium (25 mg/kg) and high dose of EZ (50 mg/kg), and TPP (50 mg/kg), treatment significantly decreased the blood HbA1c level by 35.2%, 57.8% and 36.3%, respectively. The serum insulin and glucagon concentrations in the diabetic control group were approximately 7- and 15-fold higher than that in the non-diabetic group, respectively). However, both levels were significantly decreased by medium and high dose (25 and 50 mg/kg) administration of EZ. The serum glucagon level in low-dose (12.5 mg/kg) treated group significantly increased.

Table 1 The effect of EZ administration for 6 weeks on body weight, food intake and water intake in C57BL/6J and *db/db* mice[a]

			db/db (mg/kg)			
	C57BL/6J	*db/db*[c]	TPP-50	EZ-12.5	EZ-25.0	EZ-50.0
Body weight (g)						
Initial	23.01 ± 1.35	31.92 ± 3.19	33.86 ± 2.41	30.15 ± 1.87	33.00 ± 2.05	33.92 ± 2.08
Final	27.47 ± 2.38	50.78 ± 5.67	50.94 ± 4.70	49.84 ± 3.38	49.84 ± 4.05	51.26 ± 6.09
Gain	4.31 ± 3.16	18.86 ± 7.51	17.08 ± 6.1	19.69 ± 4.46	16.83 ± 5.24	17.35 ± 7.43
Food intake (g)	2.90 ± 0.05	6.52 ± 0.14	6.57 ± 0.14	5.87 ± 0.13#	6.22 ± 0.1	6.16 ± 0.1
Water intake (ml)	4.78 ± 0.10	15.81 ± 0.76	13.76 ± 0.66	10.26 ± 0.55##	12.99 ± 0.75**	11.57 ± 0.55***
FER (%)[b]	3.30 ± 2.80	6.43 ± 2.96	5.78 ± 2.39	7.45 ± 1.95	6.01 ± 2.15	6.26 ± 3.10

EZ, Enzogenol; FER, food efficiency ratio; TPP, tea polyphenol. [a]The values are expressed as mean ± SD ($n = 8$). [b]FER (%) = (body weight gain/food intake) × 100. [c]$P < 0.001$ vs C57BL/6J group based on Tukey's test. #$P < 0.05$ and ##$P < 0.01$ vs *db/db* group based on Tukey's test.

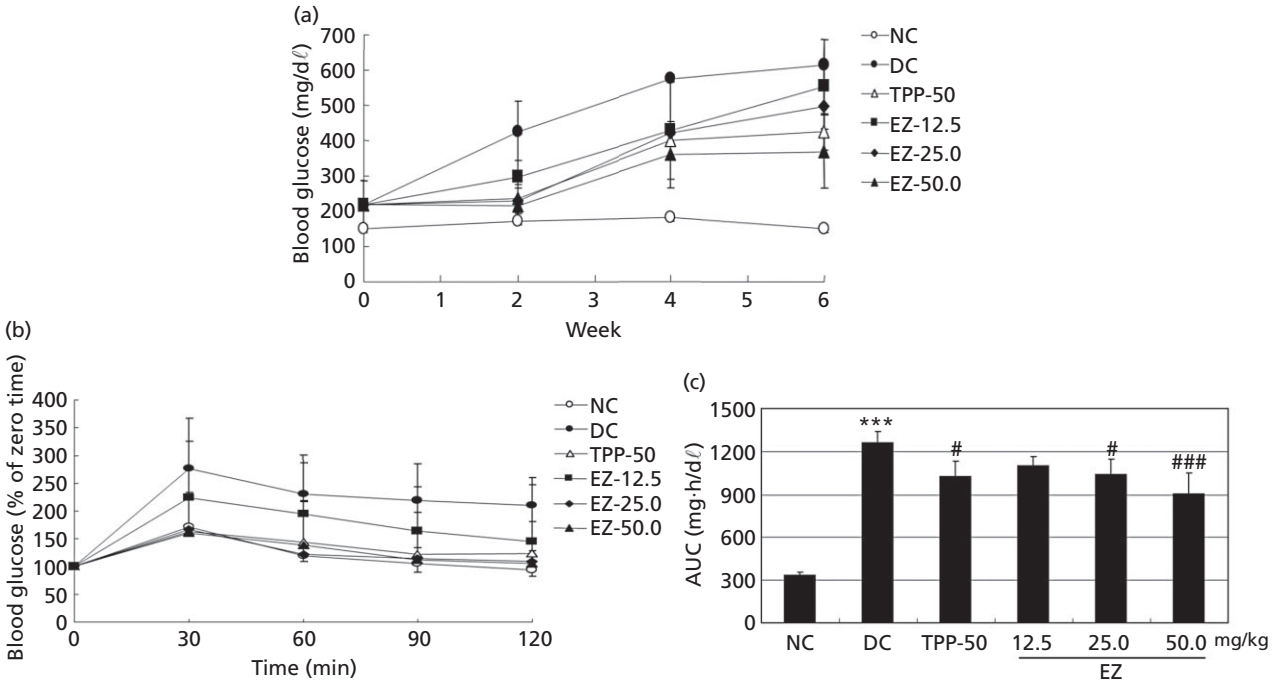

Figure 2 Blood glucose and area under curve levels in *db/db* mice. (a) Fasting glucose levels were measured after fasting for 6 h. Blood samples were collected via the tail vein. (b) The effect of administration of Enzogenol on glucose tolerance in *db/db* and C57BL/6J mice. After a 12 h fast, male mice were orally administered with glucose (3 g/kg). The blood glucose concentration was measured at the indicated time points. (c) The area under curve levels in blood glucose of oral glucose tolerance test. The values are expressed as mean ± SE ($n = 5$). ***$P < 0.001$ vs NC group; #$P < 0.05$, ##$P < 0.01$ vs DC group based on Tukey's test. NC, C57BL/6J mice (normal control); DC, *db/db* mice (diabetic control); TPP-50, tea polyphenol 50 mg/kg.

Table 2 Blood glucose levels during the 6-week experimental period in C57BL/6J and *db/db* mice[a]

Groups / Week			*db/db* (mg/kg)			
	C57BL/6J	*db/db*	TPP-50	12.5	25.0	50.0
0	151 ± 11	218 ± 68	218 ± 68	218 ± 9	218 ± 68	218 ± 68
2	173 ± 7	426 ± 85***	236 ± 41##	295 ± 124#	228 ± 37##	213 ± 52##
4	183 ± 11	574 ± 12***	401 ± 52##	429 ± 140#	421 ± 48#	359 ± 95##
6	150 ± 9	616 ± 68***	424 ± 51##	554 ± 121	495 ± 58	368 ± 103##

TPP, tea polyphenol. [a]The values are expressed as mean ± SE ($n = 5$). ***$P < 0.001$ vs C57BL/6J group based on Tukey's test. #$P < 0.05$ and ##$P < 0.01$ vs *db/db* group based on Tukey's test.

Expression of hepatic glucose-regulating enzyme and glycogen synthase

To investigate the roles of EZ in hepatic glucose-regulating proteins, we examined the expression of enzymes involved in glucose metabolism and glycogen synthesis. The diabetic control group showed reduced hepatic GCK protein level. In contrast, the expression of hepatic gluconeogenic enzymes, such as G-6-Pase and PEPCK, was higher than that of the non-diabetic control group (Figure 3). The reduced GCK expression was rescued by EZ treatment. Conversely, the increased G-6-Pase and PEPCK expression were down-regulated by EZ or TPP administration. The hepatic glycogen synthase protein level in diabetic control group was lower than that in the non-diabetic group (Figure 3). The expression of hepatic glycogen synthase in medium and high dose of EZ-treated groups was increased in a dose-dependent manner in *db/db* mice. TPP group also significantly increased in the expression of hepatic glycogen synthase.

Hepatic glucose homeostasis and lipid beta-oxidation

Recent studies demonstrated that the activity of hepatic AMPK, which is determined by phosphorylation of AMPK,

Table 3 Oral glucose tolerance test in C57BL/6J and *db/db* mice[a]

	Time (min)				
	0	30	60	90	120
C57BL/6J	100 ± 15.4	172 ± 16.1	119 ± 10.1	106 ± 16.3	94 ± 11.5
db/db	100 ± 21.5	277 ± 89.9	231 ± 68.7*	219 ± 65.2*	210 ± 50.4**
TPP-50.0	100 ± 30.3	163 ± 23.4	143 ± 19.7	122 ± 15.7	123 ± 22.1
EZ-12.5	100 ± 10.2	225 ± 9.1	195 ± 21.6	164 ± 33.5	145 ± 35.0
EZ-25.0	100 ± 18.4	167 ± 9.2	121 ± 11.7#	115 ± 18.3#	108 ± 20.3#
EZ-50.0	100 ± 34.7	160 ± 10.2##	138 ± 79.3	111 ± 52.0	105 ± 17.3#

EZ, Enzogenol; TPP, tea polyphenol. [a]The values are expressed as mean ± SE ($n = 5$). *$P < 0.05$ and **$P < 0.01$ vs C57BL/6J group based on Tukey's test. #$P < 0.05$ and ##$P < 0.01$ vs *db/db* group based on Tukey's test.

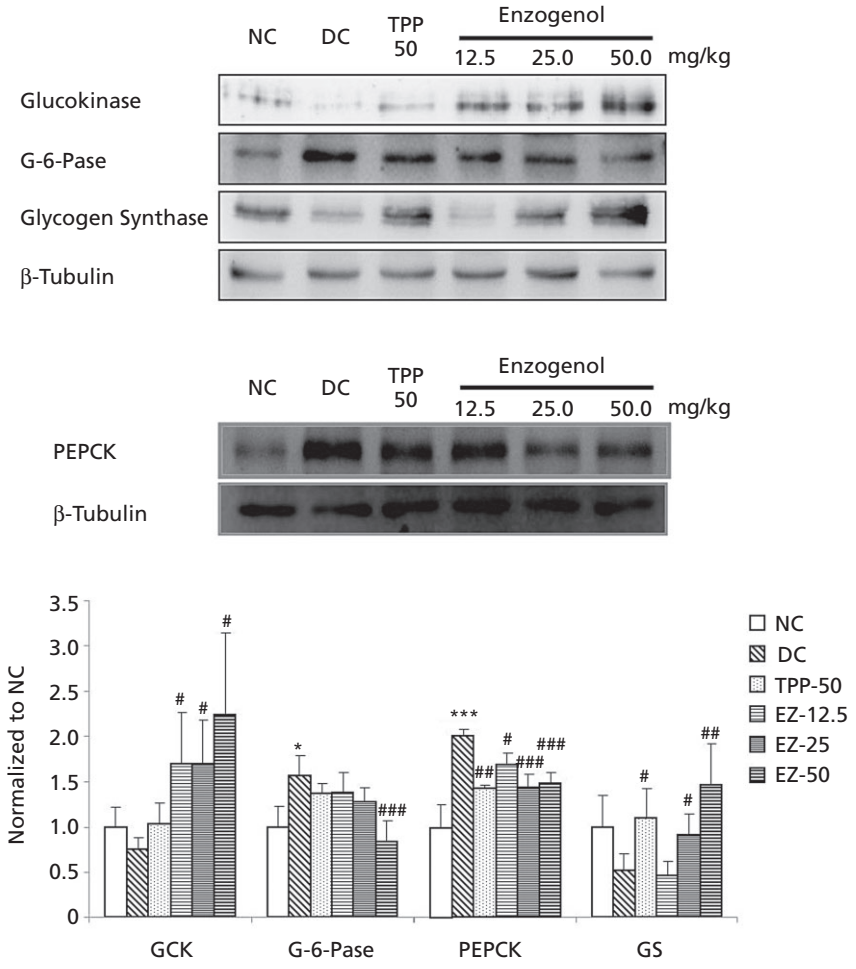

Figure 3 Expression of proteins related to hepatic glucose-regulating enzyme and glycogen synthesis. The values are expressed as mean ± SE ($n = 5$). *$P < 0.05$ and ***$P < 0.001$ vs NC group; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ vs DC group based on Tukey's test. NC, C57BL/6J mice (normal control); DC, *db/db* mice (diabetic control); TPP-50, tea polyphenol 50 mg/kg.

is dramatically reduced in *db/db* mice compared with the non-diabetic.[30] To explore more details about the effect of EZ on hepatic glucose homeostasis and beta-oxidation, we examined the activity of AMPK. There was no change of AMPK protein levels between groups. However, AMPK activity was reduced in diabetic control group compared with the non-diabetic control group. EZ administration stimulated AMPK phosphorylation dramatically and in a dose-dependent manner (Figure 4). PPAR-α and LCAD protein expressions were slightly lower in the diabetic

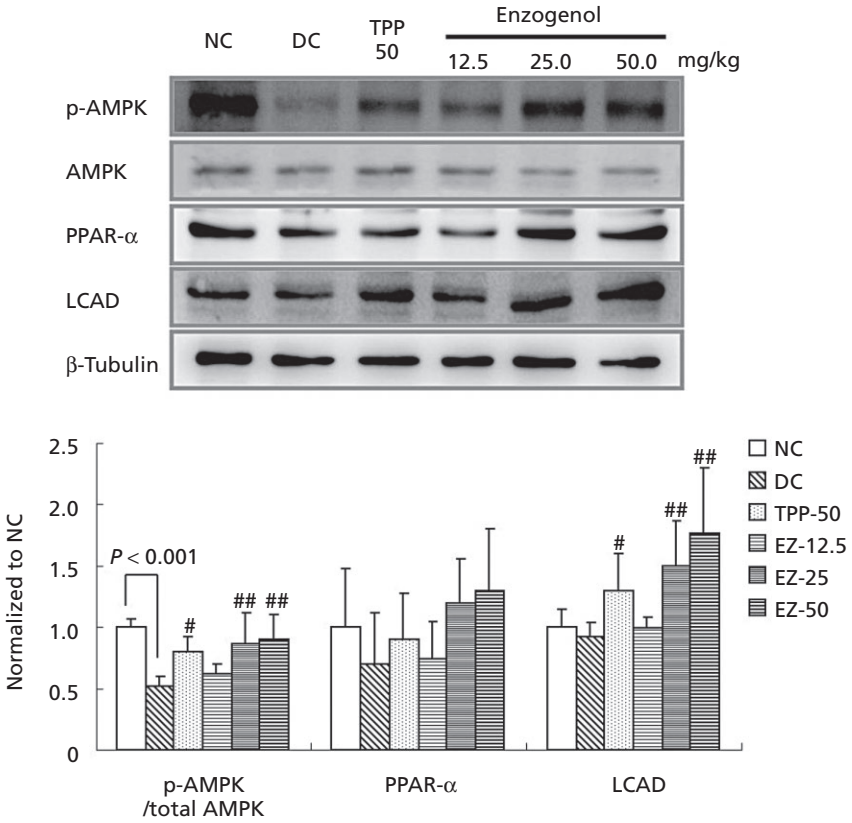

Figure 4 AMPK phosphorylation- and beta-oxidation-related protein expressions in liver tissue. The values are expressed as mean ± SE ($n = 5$). #$P < 0.05$ and ##$P < 0.01$ vs DC group based on Tukey's test. NC, C57BL/6J mice (normal control); DC, *db/db* mice (diabetic control); TPP-50, tea polyphenol 50 mg/kg.

control group compared with the non-diabetic control group (Figure 4). The reduced hepatic PPAR-α and LCAD protein levels in *db/db* mice were rescued by EZ or TPP treatment.

Hepatic lipid profiles

As EZ treatment was found to be critical for the regulation of enzymatic activity and expression of lipid metabolic proteins, we also examined the hepatic lipid levels in EZ-treated *db/db* mice (Table 3). EZ treatment resulted in significant decreases of hepatic FFA, TG and TC levels in a dose-dependent manner in *db/db* mice. Also, EZ treatment significantly and dose-dependently increased HDL-C and the ratio of HDL-C to TC that were lowered in *db/db* mice. These results are consistent with the effect of EZ on the enzymes related to hepatic beta-oxidation, such as AMPK, PPAR-α and LCAD.

Discussion

Insulin resistance is an early and sustained feature of type 2 diabetes;[12] two classes of oral antidiabetic agents, metformin and thiazolidinediones (rosiglitazone and pioglitazone), counter insulin resistance.[31] Thiazolinediones are one of the most frequently prescribed drugs to improve glycaemic control and increase insulin sensitivity in patients with type 2 diabetes. However, they have serious side effects, including hypoglycaemia, oedema, hypertension and weight gain.[32] Metformin is a synthetic biguanide and is the most widely prescribed medication for type 2 diabetes. This drug reduces hepatic glucose production, and increases peripheral glucose utilization[33,34] and insulin sensitivity.[35] Usual side effects of metformin treatment are gastrointestinal, including nausea, vomiting, diarrhoea, abdominal discomfort and flatulence, which become tolerable over time and can be decreased by administering the drug with food.[36]

Currently, there is growing interest in natural products for the treatment of diabetes mellitus. Indeed, it has been shown that several natural products, such as *Ecklonia cava*,[37] *Galega officinalis*[38] and *Momordica charantia*,[39] have significant antidiabetic effect in animal model. Moreover, recent studies have indicated that blood glucose level, hepatic lipid accumulation, adipose tissue weight and adipocyte size were significantly decreased by polyphenols

Table 4 Effects of EZ on the levels of glycosylated haemoglobin ($HbA1_C$), serum insulin and glucagon[a]

			db/db (mg/kg)			
	C57BL/6J	*db/db*[b]	TPP-50	EZ-12.5	EZ-25.0	EZ-50.0
$HbA1_C$ (%)	5.24 ± 0.25	13.40 ± 0.51	9.84 ± 0.56###	12.6 ± 0.69	9.92 ± 0.30###	8.50 ± 0.63###
Insulin (ng/ml)	2.00 ± 1.61	13.97 ± 2.26	12.90 ± 1.62	12.33 ± 4.18	5.57 ± 2.76###	4.20 ± 1.68###
Glucagon (pg/ml)	0.20 ± 0.01	3.10 ± 0.45	1.66 ± 0.72###	4.05 ± 0.21#	1.67 ± 0.98###	1.03 ± 0.29###

EZ, Enzogenol; TPP, tea polyphenol. [a]The values are expressed as mean ± SE (n = 5~8). [b]$P < 0.001$ vs normal group based on Tukey's test. #$P < 0.05$ and ###$P < 0.001$ vs *db/db* group based on Tukey's test.

Table 5 Effects of EZ on the levels of hepatic FFA, TG, TC and HDL-C[a]

			db/db (mg/kg)			
	C57BL/6J	*db/db*	TPP-50	EZ-12.5	EZ-25.0	EZ-50.0
Free fatty acid (FFA) (mEq/g tissue)	0.026 ± 0.005	0.058 ± 0.01***	0.039 ± 0.004###	0.045 ± 0.004##	0.045 ± 0.004##	0.032 ± 0.007###
Triglyceride (TG) (mg/g tissue)	3.32 ± 0.85	5.66 ± 1.42**	3.21 ± 0.86###	3.86 ± 0.34##	3.61 ± 0.70###	3.16 ± 0.74###
Total cholesterol (TC) (mg/g tissue)	3.67 ± 0.84	9.34 ± 3.14***	4.28 ± 0.64###	5.54 ± 0.98	4.02 ± 0.54###	3.78 ± 1.35###
HDL-C[b] (mg/g tissue)	2.95 ± 0.72	0.69 ± 0.20***	1.56 ± 0.45###	0.79 ± 0.13	0.82 ± 0.23	2.03 ± 0.72###
HTR[c]	80.33±18.77	7.42 ± 2.21***	36.31 ± 9.69###	14.22 ± 3.44###	20.48 ± 6.04###	53.70 ± 12.78###

EZ, Enzogenol; TPP, tea polyphenol. [a]The values are expressed as mean ± SE (n = 6 ~ 8). [b]High-density lipoprotein cholesterol. [c]HDL cholesterol to total cholesterol ratio (HTR) % = (HDL cholesterol (in mg/dℓ) = total cholesterol (in mg/dℓ)) × 100. *$P < 0.01$ and ***$P < 0.001$ vs normal group; ##$P < 0.01$ and ###$P < 0.001$ vs *db/db* group based on Tukey's test.

derived from natural product in type 2 diabetic animals without altering body weight.[40–42]

In the current study, we investigated the effect of EZ on insulin resistance and glucose homeostasis in C57BL/KsJ-*db/db* mice, an animal model for type 2 diabetes. The blood glucose (Figure 2a and 2c, Tables 4 and 5), serum insulin and glucagon levels (Table 2) in EZ-treated groups were improved after EZ treatment for 6 weeks, which suggests an enhanced rate of glucose disposal in peripheral tissues, such as the liver, muscle and adipose tissue. Glucagon plays a key role in glucose metabolism *in vivo*. Consistent with its role as a counter-regulatory hormone of insulin, glucagon raises plasma glucose levels in response to insulin-induced hypoglycaemia.[43] Glucagon induces glycogen synthase phosphorylation and inhibits glycogen synthase activity in the liver.[44–46] We also observed that EZ administration improved the glucose tolerance in *db/db* mice. EZ treatment resulted in a rapid removal of blood glucose in diabetic control group, suggesting that EZ treatment enhances insulin sensitivity (Figure 2b).

TPP, used as a positive control in this study, contains approximately 30% EGCG, which is known to have beneficial effect on type 2 diabetes. In animal and cell culture experiments, catechins have demonstrated several potentially therapeutic effects as follows: the inhibition of α-amylase in the intestine, which was presumably responsible for the resultant suppression of levels of plasma glucose and insulin,[47] EGCG decreases glucose production of H4IIE rat hepatoma cells; furthermore, EGCG mimics insulin, increases tyrosine phosphorylation of the insulin receptor and the insulin receptor substrate, and reduces gene expression of the PEPCK,[48] and suppresses cytokine-induced pancreatic beta-cell damage in vitro.[49]

To gain insight into the molecular mechanisms underlying the effects described above, we investigated genes that are likely to be involved in glucose homeostasis and lipid metabolism. Hepatic GCK plays a major role in controlling blood glucose homeostasis, and its activity is low in diabetes.[50] G-6-Pase is a key enzyme controlling hepatic gluconeogenesis and glucose output in liver and is normally suppressed by the action of insulin.[51] PEPCK catalyses the conversion of oxaloacetate into phospho*enol*pyruvate, an early and rate-limiting step in the pathway of hepatic gluconeogenesis.[15] Due to their strategic positions in liver glucose metabolism, both of these enzymes are supposed to be the target of important regulatory mechanisms of hepatic glucose production.[52] Our studies demonstrated that EZ administration showed hypoglycaemic effect by stimulating GCK activity and inhibiting G-6-Pase and PEPCK activity, which are hepatic glucose-regulating enzymes in the liver. The expression of hepatic GCK and glycogen synthase was increased remarkably, whereas those

of G-6-Pase and PEPCK were significantly decreased in a dose-dependent manner in the EZ-treated groups in *db/db* mice (Figure 3). Thus, it appears that EZ improves hepatic glucose metabolism through an increase in GCK and glycogen synthase activity, and a decrease in gluconeogenic enzyme activity (i.e. G-6-Pase and PEPCK).

We showed that EZ administration promotes activation of AMPK, equivalent to TPP administration (Figure 4). AMPK activation serves to (1) inhibit hepatic fatty acid and cholesterol synthesis, and (2) stimulate fatty acid oxidation in the liver and muscle.[53,54] Our studies also demonstrated that EZ and TPP treatment increased the protein expression of LCAD (Figure 4). The peroxisome proliferator-activated receptor (PPAR)α is a nuclear receptor mainly involved in the regulation of lipid levels. PPAR-α agonists is known to reduce both intrahepatic and intramuscular TG content and improve insulin sensitivity in rodents.[55–57]

As shown in Figure 4, PPAR-α expression, which was reduced in diabetic control, was rescued by EZ treatment. These results are consistent with the decrease of hepatic TGs and FFA contents in EZ-treated group (Table 3). In addition, as shown in Table 3, TC level in diabetic control group was significantly decreased by EZ administration in a dose-dependent manner. The HDL-C/TC ratio was also significantly improved in the EZ-treated group (Table 3). Noticeably, EZ treatment did not induce further increase of body weight, which is observed in some synthetic anti-diabetic drug-treated patients.[58] Compared with non-treated control group, we found decrease of body weight gain in EZ-treated *db/db* mice (Table 1), although it was not statistically significant (Table 1). To determine whether EZ has pharmaceutical possibility, it is necessary to examine whether other organs, such as white adipose tissue and skeletal muscle, regulate lipids or insulin signal pathway.

Conclusions

In this study, EZ treatment significantly lowered the levels of glucose, insulin and glucagon in serum, and improved glucose tolerance. EZ appears to have hypoglycaemic effects by modulating the expression of hepatic glucose-regulating enzymes, GCK, G-6-Pase and PEPCK, and enzymatic activity of AMPK in the liver. These results demonstrate that EZ supplementation could exert beneficial effects on type 2 diabetes.

Declarations

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