

Brief communication

## Dietary supplementation with cacao liquor proanthocyanidins prevents elevation of blood glucose levels in diabetic obese mice

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### Abstract

**Objective:** Effective approaches should be established to prevent the onset of type 2 diabetes mellitus, which has been increasing in developed countries. The present study examined whether dietary supplementation with cacao liquor proanthocyanidins (CLPr) could prevent elevation of blood glucose levels in mice with diabetes mellitus and obesity.

**Methods:** C57BL/KsJ-db/db (db/db) diabetic obese mice and C57BL/KsJ-db/+m (db/+m) control mice were fed a diet containing 0% w/w CLPr (0% CLPr), 0.5% w/w CLPr (0.5% CLPr), or 1.0% w/w CLPr (1.0% CLPr) from age 3 wk to age 6 wk. Levels of blood glucose were measured at 4 and 5 wk of age. The animals were sacrificed and the levels of blood glucose and fructosamine were measured at 6 wk of age.

**Results:** The levels of blood glucose and fructosamine were higher in the db/db mice than in the db/+m mice fed a diet containing 0%, 0.5%, or 1.0% CLPr. In the db/+m mice, the levels of blood glucose or fructosamine were not significantly different across animals fed 0% CLPr, 0.5% CLPr, and 1.0% CLPr. In the db/db mice, however, a diet containing 0.5% or 1.0% CLPr decreased the levels of blood glucose and fructosamine compared with that containing 0% CLPr without significant effects on body weights or food consumption.

**Conclusion:** Dietary supplementation with CLPr can dose-dependently prevent the development of hyperglycemia in diabetic obese mice. The dietary intake of food or drinks produced from cacao beans might be beneficial in preventing the onset of type 2 diabetes mellitus. © 2007 Elsevier Inc. All rights reserved.

### Keywords:

Type 2 diabetes mellitus; Obesity; Cacao liquor proanthocyanidins; Hyperglycemia; Antioxidation

### Introduction

Recently, the prevalence of type 2 diabetes mellitus has reached epidemic levels in Western countries. However, effective control of the onset of diabetes mellitus and its complications has not been established. Oxidative stress plays an important role in the pathogenesis of different diseases [1]. Diabetes mellitus in experimental animals and

humans is associated with reductions in antioxidants such as ascorbic acid,  $\alpha$ -tocopherol, and glutathione, suggesting the critical role of oxidative stress in its pathogenesis.

Flavonoids are natural polyphenolic antioxidants present in a wide range of plant foods. Many dietary flavonoids protect against oxidative damage at least in part by directly neutralizing reactive oxidants. We previously reported that rosmarinic acid, a naturally occurring polyphenol with antioxidative and anti-inflammatory activities, inhibits lung inflammation and oxidative stress induced by diesel exhaust particles [2].

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In contrast, cacao liquor proanthocyanidins (CLPr), ingredients of chocolate and cocoa, are rich in polyphenols, including catechins and their oligomers linked by C4 and C8 bound as B-type proanthocyanidins. We also reported that these polyphenols have antioxidative activity at least in vitro [3]. It has been reported that cacao or chocolate can reduce the risk of cardiovascular illness including atherosclerosis, hypertension, and platelet activity possibly by its antioxidative property [4–7]. Furthermore, a clinical trial in healthy human volunteers has indicated that daily intake of cocoa powder rich in proanthocyanidins reduces the susceptibility of low-density lipoproteins to oxidation [8]. However, there is no report on the efficacy of CLPr on diabetes mellitus with obesity.

The present study was designed to determine the effects of dietary supplementation with CLPr on type 2 diabetes mellitus.

## Materials and methods

### Animals

Three-week-old female C57BL/KsJ-db/db Jcl (db/db) mice weighing 10–14.7 g and C57BL/KsJ-db/+m Jcl (db/+m) mice weighing 8.6–14.5 g as normal controls were supplied from Japan Clea Co. (Tokyo, Japan). The mice were housed in an animal facility that was maintained at 24–26°C with 55–75% humidity and a 14-/10-h light/dark cycle; water was given ad libitum. The studies followed the National Institutes of Health guidelines for the experimental use of animals. The institutional review board approved the animal studies.

### Preparation of CLPr

The CLPr were prepared from cacao solution as previously reported [9] and included total polyphenols of 72.32%, consisting of catechin (2.49%), epicatechin (5.89%), procyanidin B2 (3.93%), procyanidin C1 (2.38%), cinnamtannin A2 (3.17%), and galactopyranosyl-ent-(–)-epicatechin-(–)-epicatechin (0.48%). The total polyphenol content was evaluated by the Prussian blue method [10] using epicatechin as the standard. Catechins and proanthocyanidins were determined by reversed-phase high-performance liquid chromatography/mass spectrometry [9].

### Study protocol

The db/db mice and db/+m mice were randomly divided into six experimental groups.

The animals were fed the American Institute of Nutrition 93 diet containing 0% w/w CLPr (0% CLPr), 0.5% w/w CLPr (0.5% CLPr), or 1% w/w CLPr (1% CLPr) in powder form for 3 wk. Postprandial blood was collected from the

tail vein and glucose was measured with a blood glucose test meter (Arkray, Inc., Kyoto, Japan) at 4 and 5 wk of age. Body weight was continuously measured. Mice were placed in cages with free access to food. Food consumption was estimated by measuring the weight of cases containing diet powder every day for 3 wk. Because the cases were a hanging type (Toyoriko Co., Aichi, Japan), a minimum of the diet was wasted.

At 6 wk of age, the animals were exsanguinated under deep anesthesia with diethyl ether; body weights and kidney weights were measured. Plasma samples were obtained for postprandial blood glucose, fructosamine, alanine aminotransferase, serum urea nitrogen (BUN), and creatinine.

### Statistical analysis

Data were reported as mean  $\pm$  SEM. Differences among groups were determined using analysis of variance (Statview 4.0, Abacus Concepts, Inc., Berkeley, CA, USA). If differences among groups were significant ( $P < 0.05$ ), Fisher's protected least significant difference test was used to distinguish between pairs of groups.

## Results

### Effects of CLPr on body weights and food consumption

Body weights were greater in the db/db mice than in the db/+m mice fed a diet containing each supplementation with CLPr ( $P < 0.01$ ). In both strains of mice, body weights were not significantly different between supplementation with 0% CLPr, 0.5% CLPr, and 1.0% CLPr at 4, 5, and 6 wk of age (Table 1). Throughout the study period, food consumption was greater in the db/db mice than in the db/+m mice ( $P < 0.01$  at 4 wk of age,  $P < 0.05$  for mice fed 1.0% CLPr at 5 wk of age; Table 1). In both strains of mice, however, food consumption was not significantly different between supplementation with 0% CLPr, 0.5% CLPr, and 1.0% CLPr throughout the study period (Table 1).

### Effects of CLPr on blood glucose and fructosamine

The levels of blood glucose ( $P < 0.01$  for mice fed 0% CLPr at 4, 5, and 6 wk of age and mice fed 0.5% or 1.0% CLPr at 5 and 6 wk of age,  $P < 0.05$  for mice fed 0.5% CLPr at 4 wk of age) and fructosamine ( $P < 0.01$  for mice fed 0% or 0.5% CLPr) were higher in the db/db mice than in the db/+m mice (Fig. 1a–d). In the db/+m mice, the levels of blood glucose and fructosamine were not significantly different between the mice fed 0%, 0.5%, and 1.0% CLPr throughout the experimental period (Fig. 1a–d). In the db/db mice fed 1.0% CLPr, however, the levels of blood glucose at 4 and 5 wk of age and of fructosamine at 6 wk of age were significantly lower than in those fed 0% CLPr ( $P < 0.01$ ; Fig. 1a, b, d). Also, supplementation with 0.5%

Table 1  
Effects of CLPr on body weights and food consumption\*

	db/+m			db/db		
	0% CLPr	0.5% CLPr	1.0% CLPr	0% CLPr	0.5% CLPr	1.0% CLPr
No. of mice	15	15	15	15	14	15
Body weights (g)						
4 wk of age	13.2 ± 0.22	13.8 ± 0.33	13.1 ± 0.20	15.6 ± 0.34 <sup>‡</sup>	15.4 ± 0.23 <sup>‡</sup>	15.0 ± 0.24 <sup>‡</sup>
5 wk of age	18.8 ± 0.34	18.8 ± 0.38	18.4 ± 0.34	21.9 ± 0.41 <sup>‡</sup>	21.6 ± 0.26 <sup>‡</sup>	21.1 ± 0.29 <sup>‡</sup>
6 wk of age	20.8 ± 0.54	20.8 ± 0.52	20.3 ± 0.60	25.6 ± 0.48 <sup>‡</sup>	25.5 ± 0.30 <sup>‡</sup>	25.0 ± 0.38 <sup>‡</sup>
Food consumption (g/d)						
4 wk of age	2.99 ± 0.08	3.18 ± 0.08	3.10 ± 0.06	3.90 ± 0.15 <sup>‡</sup>	3.55 ± 0.21 <sup>‡</sup>	3.65 ± 0.15 <sup>‡</sup>
5 wk of age	3.97 ± 0.36	4.17 ± 0.37	3.11 ± 0.33	4.74 ± 0.45	4.83 ± 0.62	4.52 ± 0.45 <sup>†</sup>
6 wk of age	2.76 ± 0.20	3.08 ± 0.26	3.01 ± 0.23	4.50 ± 1.01	4.33 ± 0.73	4.32 ± 0.82
ALT at 6 wk of age (IU/L)	24 ± 3.9	24 ± 4.9	29 ± 4.4	486 ± 48.8 <sup>‡</sup>	435 ± 28.7 <sup>‡</sup>	432 ± 46.3 <sup>‡</sup>
BUN at 6 wk of age (mg/dL)	15.7 ± 0.64	17.4 ± 0.82	15.4 ± 0.76	26.4 ± 1.34 <sup>‡</sup>	25.2 ± 0.86 <sup>‡</sup>	21.9 ± 0.90 <sup>‡  </sup>
Creatinine at 6 wk of age (mg/dL)	0.87 ± 0.036	0.69 ± 0.033 <sup>  </sup>	0.62 ± 0.030 <sup>  </sup>	0.89 ± 0.025	0.88 ± 0.028 <sup>‡</sup>	0.79 ± 0.023 <sup>‡§</sup>
Kidney weights (g)	0.103 ± 0.003	0.106 ± 0.003	0.108 ± 0.003	0.130 ± 0.04 <sup>‡</sup>	0.122 ± 0.02 <sup>‡</sup>	0.120 ± 0.03 <sup>‡§</sup>

ALT, alanine aminotransferase; BUN, serum urea nitrogen; CLPr, cacao liquor proanthocyanidins; db/db, diabetic mice; db/+m, non-diabetic mice

\* The db/db and db/+m mice were fed a diet containing 0%, 0.5%, or 1.0% CLPr for 3 wk. The db/db mice and db/+m mice were randomly divided into six experimental groups. Body weights and food consumption were continuously measured from age 4 wk to 6 wk. Blood samples were collected after exsanguination at 6 wk of age, and plasma levels of ALT, BUN, and creatinine and kidney weights were measured. Values are presented as mean ± SEM.

<sup>†</sup>  $P < 0.05$  versus db/+m mice fed a diet containing an identical dose of CLPr.

<sup>‡</sup>  $P < 0.01$  versus db/+m mice fed a diet containing an identical dose of CLPr.

<sup>§</sup>  $P < 0.05$  versus an identical strain fed a diet containing 0% CLPr.

<sup>||</sup>  $P < 0.01$  versus an identical strain fed a diet containing 0% CLPr.

CLPr significantly decreased the levels of glucose at 5 wk of age compared with supplementation with 0% CLPr ( $P < 0.01$ ; Fig. 1b).

#### Effects of CLPr on biochemical parameters and kidney weights

In the overall trends, the levels of alanine aminotransferase, BUN, creatinine, and kidney weights were greater in the db/db mice than in the db/+m mice fed diets containing supplementation with CLPr ( $P < 0.01$  but for creatine in mice fed diet containing 0% CLPr; Table 1). In the db/+m mice, supplementation with 0.5% or 1.0% CLPr significantly decreased the levels of creatinine ( $P < 0.01$ ) compared with supplementation with 0% CLPr (Table 1). In the db/db mice fed a diet containing 1.0% CLPr, the levels of BUN ( $P < 0.01$ ), creatinine ( $P < 0.05$ ), and kidney weights ( $P < 0.05$ ) were significantly lower than in those fed a diet containing 0% CLPr (Table 1).

#### Discussion

The present study has demonstrated that a diet containing CLPr decreases blood glucose levels in diabetic obese mice in a dose-dependent fashion.

The db/db mice are a useful model for diabetes mellitus with obesity and insulin resistance [11]. In this study, a diet containing CLPr decreased the levels of blood glucose and

fructosamine compared with a diet without CLPr in the db/db mice. To our knowledge, this is the first study to report that CLPr can prevent aggravation of type 2 diabetes mellitus. Importantly, food consumption or weight gain was not significantly different among mice fed diets containing 0%, 0.5%, and 1.0% CLPr.

It has been reported that proanthocyanidins in cacao including monomeric (+)-catechin and (–)-epicatechin, dimeric procyanidin B2, trimeric procyanidin C1, and tetrameric cinnamtannin have antioxidative activity [12,13]. Polyphenol-rich dark chocolate improves insulin sensitivity [6] or reduces insulin resistance [14], whereas reactive oxygen species have a causal role in insulin resistance [15]. In contrast, obesity is a causative factor in the development of insulin resistance and/or the metabolic syndrome. Increased oxidative stress in accumulated fat is an important pathogenic mechanism of obesity-associated metabolic syndrome [16]. In this study, the decreased levels of blood glucose may be involved in improvement of insulin resistance by antioxidative effects of CLPr. We examined the levels of ascorbic acid in urine, but there was no significant relation across the experimental groups (data not shown). In future, other antioxidants should be examined.

In this study, CLPr extract decreased the levels of BUN, creatinine, and kidney weights compared with 0% CLPr in the db/db mice. It has been reported that advanced glycation end products accumulate in the tissues of patients with diabetes and chronic renal failure [17]. Alternatively, carbonyl and oxidative stress play a key role in the pathogenesis of diabetes and/or its complications via advanced gly-

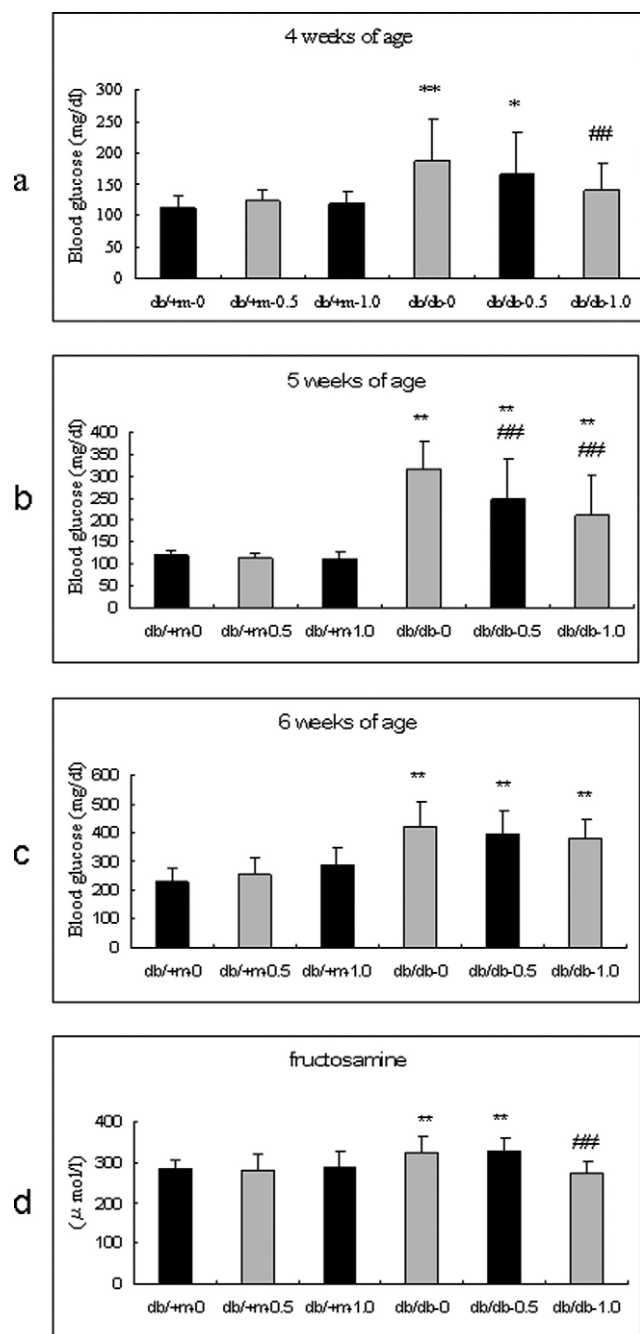


Fig. 1. Diabetic (db/db) and non-diabetic (db/+m) mice were fed diets containing 0%, 0.5%, or 1.0% cacao liquor proanthocyanidins (CLPr) for 3 wk. Db/db mice and db/+m mice were randomly divided into six experimental groups. The levels of blood glucose were measured at 4 wk (a) and 5 wk (b) of age. Blood samples were collected after exsanguination at 6 wk of age, and plasma levels of glucose (c) and fructosamine (d) were measured. \* $P < 0.05$  versus db/+m mice fed diets containing identical doses of CLPr; \*\* $P < 0.01$  versus db/+m mice fed diets containing identical doses of CLPr; \*\*\* $P < 0.01$  versus an identical strain fed a diet containing 0% CLPr. Values are means  $\pm$  SEMs for each group. db/+m-0, db/+m-0% CLPr group; db/+m-0.5, db/+m-0.5% CLPr group; db/+m-1.0, db/+m-1.0% CLPr group; db/db-0, db/db-0% CLPr group; db/db-0.5, db/db-0.5% CLPr group; db/db-1.0, db/db-1.0% CLPr group.

cation end products pathway [18]. In contrast, Stefanovic et al. [19] reported that grape seeds, which contain antioxidative bioflavonoids, afford a significant morphologic improvement and amelioration in kidney function [19]. In our investigation, immunohistologic assessment using anti-8OHdG or anti-4-hydroxy-2-nonenal antibody demonstrated that CLPr suppressed the enhancement of carbonyl and oxidative stress in the kidney of db/db mice (data not shown). Thus, prevention of carbonyl and oxidative stress should be important for renal dysfunction.

In addition, procyanidins and/or flavonol-rich foods such as cocoa reportedly inhibit angiotensin converting enzyme activity [20]. It is also possible that the preventive effects of CLPr on renal damage are mediated at least in part through angiotensin converting enzyme inhibition and/or antioxidative activity as the suppressor of advanced glycation end production.

A 1% concentration in the diet used in the present study would correspond to about 5 g of polyphenols per day for a human subject, or about 2.5 kg of chocolate. However, Grassi et al. [14] tested the effect of flavonol-rich dark chocolate on oral glucose tolerance tests in patients with essential hypertension. The insulin resistance was ameliorated by the intake of 100 g of dark chocolate per day (containing 88 mg of flavonols). On the basis of the previous report and the present results, chocolate, cocoa, and other functional foods that contain more CLPr may be recommended to obtain the benefits of cacao proanthocyanidins.

In conclusion, supplementation with CLPr could dose-dependently suppress hyperglycemia in diabetic obese mice. Dietary intake of a polyphenol-rich food such as cacao beans might be beneficial in preventing the onset of type 2 diabetes mellitus.

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