Ingestion of Proanthocyanidins Derived from Cacao Inhibits Diabetes-Induced Cataract Formation in Rats

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Proanthocyanidins derived from cacao (CLP) have various antipathophysiological functions. We have tested whether dietary supplementation with CLP prevents cataract formation in rats with diabetes induced by streptozotocin (STZ), using histological, histochemical, and biochemical analyses. Starting at 7 days after the streptozotocin challenge, the animals were fed either a normal diet or a diet containing 0.5% w/w CLP over 10 weeks. There were no significant differences in plasma and urine glucose concentrations, plasma fructose amines, and plasma thiobarbituric reactive substances (TBARS) between the two dietary groups. Antioxidant status as assessed by measuring lipid peroxide production in plasma in response to azocompounds was lower in the STZ-rats fed control diet than in animals fed CLP. Opacity was first detected in the lenses of the control dietary group 5 weeks after STZ injection and cataracts had developed in the majority of these animals by 10 weeks. These changes were rarely seen in the STZ/CLP diet group. Histological examinations of the eyes of the STZ-treated normal diet group revealed focal hyperplasia of the lens epithelium and liquefaction of cortical fibers. There were similar but considerably less severe changes in the animals fed CLP. Hydroxynonenal (HNE), a marker of oxidative stress, was detected immunohistochemically in the lenses of the STZ-treated normal diet group, but not of those receiving CLP. Our findings suggest that CLP inhibits diabetes-induced cataract formation possibly by virtue of its antioxidative activity. Exp Biol Med 229:33–39, 2004

Key words: proanthocyanidin; cacao; diabetes-induced cataract; HNE; oxidative stress

Oxygen radicals play an important role in the origin of a wide range of diseases (1–5). Diabetes mellitus in both experimental animal models and humans is associated with marked reductions in the levels of plasma antioxidants such as α-tocopherol, ascorbic acid, uric acid, and glutathione causing lowered plasma antioxidative capacity. Various biochemical imbalances contribute to the development of diabetic micro- and macrovascular complications, including atherosclerosis. Among these complications, hyperglycemia-induced cataract formation is thought to be caused by the accumulation of sorbitol (6, 7). Recently, many studies have suggested that oxidative stress also has a crucial role in diabetes-induced cataract formation (8–10). This has led researchers to investigate whether the vascular complications of diabetes, including cataract formation, can be reduced by dietary supplementation with antioxidants (11–13).

Cacao liquor (CLP), an ingredient of chocolate and cocoa, is rich in polyphenols (14–16), including catechins and their oligomers linked by C4, C8 bonds as B-type proanthocyanidins (17). We have reported previously that these polyphenols have potent *in vitro* antioxidative activity (18, 19). The procyanidin fraction of CLP reduced oxidative stress in vitamin E-deficient rats (20) and hypercholesterolemic rabbits (21). In addition, a clinical trial in healthy human volunteers found that daily intake of cocoa powder rich in proanthocyanidins reduced the susceptibility of low-density lipoproteins to oxidation (22).

Here we have evaluated the effect of CLP on cataract formation, one of the major complications of both Type I and II diabetes, using the streptozotocin-induced diabetic rat as a model of human Type I diabetes.

Material and Methods

Materials. Streptozotocin, (−)-epicatechin, (+)-catechin, theobromin, caffeine, and fructoseamine calibrator

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Table 1. Composition of Polyphenol Fraction Derived from Cacao Liquor (CLP)

	Concentration (w/w%)
(+)-Catechin	0.46
(−)-Epicatechin	1.83
Procyanidin B2	1.69
Procyanidin C1	2.37
Cinnamtannin A2	2.01
Caffeine	ND[a]
Theobrimine	ND

[a] ND, not determined.

were purchased from Sigma (St. Louis, MO). Lipid peroxide analysis kits (Determiner LPO) were obtained from Kyowa Medics Ltd. (Japan) and antihydroxynonenal antibody from Calbiochem (San Diego, CA).

The crude polyphenol fraction of CLP was prepared by the method we reported previously (18). Briefly, fat was removed from the liquor with n-hexane, followed by extraction with 80% (v/v) ethanol. The extracts were passed through a chromatographic column (Diaion HP 2MG; Mitsubishi, Kasei, Japan) and contaminants, including caffeine and theobromine, were removed by washing the column with 20% (v/v) ethanol. A crude polyphenol fraction was eluted with 80% ethanol, concentrated, and freeze dried. The total polyphenol content of the preparation was measured using the Prussian blue method with epicatechin as standard (23). Catechins, procyanidins, and xanthine derivatives were analyzed by high-performance liquid chromatography (HPLC) as described previously (24). The analysis of CLP is shown in Table 1.

Animals. Eight-week-old male Sprague-Dawley rats from Clea, Japan, were housed in a controlled environment at 23°C and 55% humidity under a 12:12-hr light:dark cycle. The Animal Committee of Meiji Seika Kaisha Ltd. Health and Bioscience Laboratory approved the study, and the animals were cared for in conformity with established guidelines.

Experimental Procedures

The animals were fed the AIN-76 diet for 4 days. Food was withdrawn 16 hrs before treatment. Rats received a single intraperitioneal injection of streptozotocin (STZ; 40 mg/kg) dissolved in 0.1 *M* citrate buffer. An equal number of age-matched nondiabetic animals was selected as untreated controls. Seven days after the STZ challenge, blood and urine glucose levels were determined and animals with elevated levels divided randomly into two groups. The first group was fed AIN-76 diet for 10 weeks (STZ/normal diet group) while the second group received AIN-76 diet containing 0.5% CLP for this period (STZ/CLP diet group). Blood was collected from the tail vein of all the animals and 24-hr urines collected using a metabolic cage each week. At the end of the study, the animals were anesthetized with diethyl ether, blood was collected from the abdominal vein with heparinized syringes, and the eyes removed for examination. Tissues and plasma samples were kept at −80°C.

Analyses. Plasma and urine glucose concentrations were determined with a glucose assay kit (Wako Pure Chemical Industries, Tokyo, Japan). Thiobarbituric acid reactive substances (TBARS) in plasma were measured by the method of Ohkawa *et al.* (25) with tetraethoxypropane as standard. Fructoseamine in plasma was measured by the method of Johnson *et al.* (26), and the antioxidative status of the plasma was assessed by the method of Lotito and Fraga (27). Briefly, a reaction mixture containing plasma and 25 m*M* 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) was incubated at 37°C for 60 mins and the lipid peroxide concentration in the oxidized plasma determined with a commercial kit (Determiner LPO; Kyowa Medics Ltd., Tokyo, Japan).

Histological Analyses. The eyes were fixed in 3% buffered formalin and embedded in paraffin. Sections 5 μm thick were prepared and fixed to slides, deparaffinized, and stained with hematoxylin and eosin (HE) to permit histological examination of cortical fibers and lenses. The production of hydroxynonenal (HNE) was detected immunohistochemically with anti-HNE polyclonal antibody. Briefly, deparaffinized slides were placed in a blocking reagent containing H_2O_2 for 45 secs in order to quench endogenous peroxidase. The sections were then blocked with 10% nonimmune goat serum for 1 hr followed by incubation for 18 hrs at 4°C in a moist chamber with a 1:400 dilution of anti-HNE. This was followed by addition of a biotinylated secondary antibody and streptavidin-peroxidase conjugate. The slides were then incubated with 3,3-diaminobenzidine tetrahydrochloride to detect HNE production and counterstained with hematoxylin.

The histological appearance of the lenses was evaluated in 20 random areas (0.0625 mm^2/area) at ×400 magnification. The slides were graded blind without knowledge of the experimental design. Cortical fiber liquefaction was graded as follows: no observable changes, slight changes, moderate changes, and severe changes. Focal hyperplasia of lenses was graded as follows: no observable changes; mild, faint changes; moderate changes; and obvious changes.

Statistical Analysis. The analyses were carried out with SPSS statistical software and the data expressed as means ± standard deviations. When analysis of variance (ANOVA) revealed significant changes of $P < 0.05$, the data were further analyzed by Tukey's multiple range test. Results of the histological examination were analyzed by the chi-square test.

Results

Mean body weight at 5 and 10 weeks in the diabetic rats was significantly lower than in animals not injected with STZ (Table 2). There was no difference in body weight

Table 2. Cacao Proanthocyanidins Did Not Effect Body Weight, Plasma and Urine Glucose, and Plasma Fructose Amine Levels in STZ Rat[a]

	Untreated control (n = 8)		STZ/normal diet (n = 9)		STZ/CLP diet (n = 8)	
Body weight (g)						
Before treatment	293.42 ± 5.87	a	289.63 ± 4.10	a	290.88 ± 3.15	a
5 Weeks	442.86 ± 12.08	a	289.51 ± 10.86	b	310.11 ± 7.75	b
10 Weeks	497.27 ± 12.24	a	302.11 ± 7.19	b	322.94 ± 6.75	b
Plasma glucose (mg/dl)						
Before treatment	92.8 ± 2.3	a	92.2 ± 2.4	a	89.3 ± 4.0	a
5 Weeks	146.3 ± 6.0	a	640.3 ± 40.1	b	651.3 ± 33.2	b
10 Weeks	144.4 ± 7.7	a	732.6 ± 40.1	b	685.1 ± 46.9	b
Glucose excretion amount in urine (mg/day)						
Before treatment	6.6 ± 1.4	a	5.1 ± 0.8	a	7.1 ± 1.1	a
5 Weeks	10.5 ± 2.1	a	26732.8 ± 1821.5	b	22176.8 ± 1730.7	b
10 Weeks	24.3 ± 8.2	a	21254.7 ± 1177.7	b	18028.5 ± 1135.2	b
Plasma fructose amine (m*M*)						
Before treatment	1.023 ± 0.019	a	1.121 ± 0.030	a	0.997 ± 0.022	a
5 Weeks	1.210 ± 0.028	a	1.678 ± 0.032	b	1.685 ± 0.030	b
10 Weeks	1.210 ± 0.033	a	1.781 ± 0.064	b	1.855 ± 0.082	b

[a] Values are means ± SE. Values within a row not sharing the same letters are significantly different, $P < 0.05$.

between diabetic rats fed diets with and without 0.5% CLP. The concentrations of plasma and urinary glucose were markedly elevated in the STZ-treated animals regardless of the presence or absence of CLP in the diet. Likewise, fructosamine concentration, an index of plasma glucose levels during the preceding 2 weeks, increased to the same extent in both the STZ treatment groups.

There were no significant differences in plasma TBARS levels at 5 and 10 weeks after administration of STZ (Table 3). However, AAPH-induced plasma lipid peroxide levels increased substantially in the STZ/normal diet group, and this increase was significantly inhibited at 10 weeks by CLP supplementation.

A representative photo of eyes taken from the experimental animals is shown in Figure 1a. In contrast with the lenses of untreated control rats, there is advanced lens opacity in the diabetic rats not receiving CLP. Histological sections of an eye that developed cataract and stained with HE are shown in Figure 1b. No changes were apparent in the lens fiber and epithelial cells in the posterior region in the nondiabetic animals. In the STZ-treated normal diet group, liquefaction of cortical fibers and focal hyperplasia of the lens epithelium were evident, whereas the eyes of the CLP diet group showed only slight swelling and granular degeneration of fibers. Table 4 summarizes the histopathological findings. Moderate to severe changes characterized by liquefaction were present in the lens fibers of more than half the animals in the STZ/normal diet group. Hyperplasia of the lens epithelium was also frequently observed in these animals, and CLP supplementation substantially reduced its prevalence.

Production of HNE in the lens, detected immunohistochemically, is illustrated in Figure 1c. Light-brown staining indicative of the presence of HNE was not detected

Table 3. Cacao Proanthocyanidins Decrease AAPH-Induced Production of Lipid Peroxides but Not TBARS in Plasma of STZ Rat[a]

	Untreated control (n = 8)		STZ/normal diet (n = 9)		STZ/CLP diet (n = 8)	
TBARS (nmol/ml)						
Before treatment	6.36 ± 0.31	a	6.44 ± 0.23	a	6.70 ± 0.55	a
5 Weeks	9.78 ± 0.63	a	7.85 ± 0.50	a	6.09 ± 0.53	a
10 Weeks	8.39 ± 0.40	a	6.91 ± 0.29	a	8.22 ± 1.01	a
LPO production induced by AAPH (m*M*)						
10 weeks	41.5 ± 2.55	a	65.3 ± 3.15	b	51.2 ± 2.88	a

[a] Values are means ± SE. Values within a row not sharing the same letters are significantly different, $P < 0.05$.

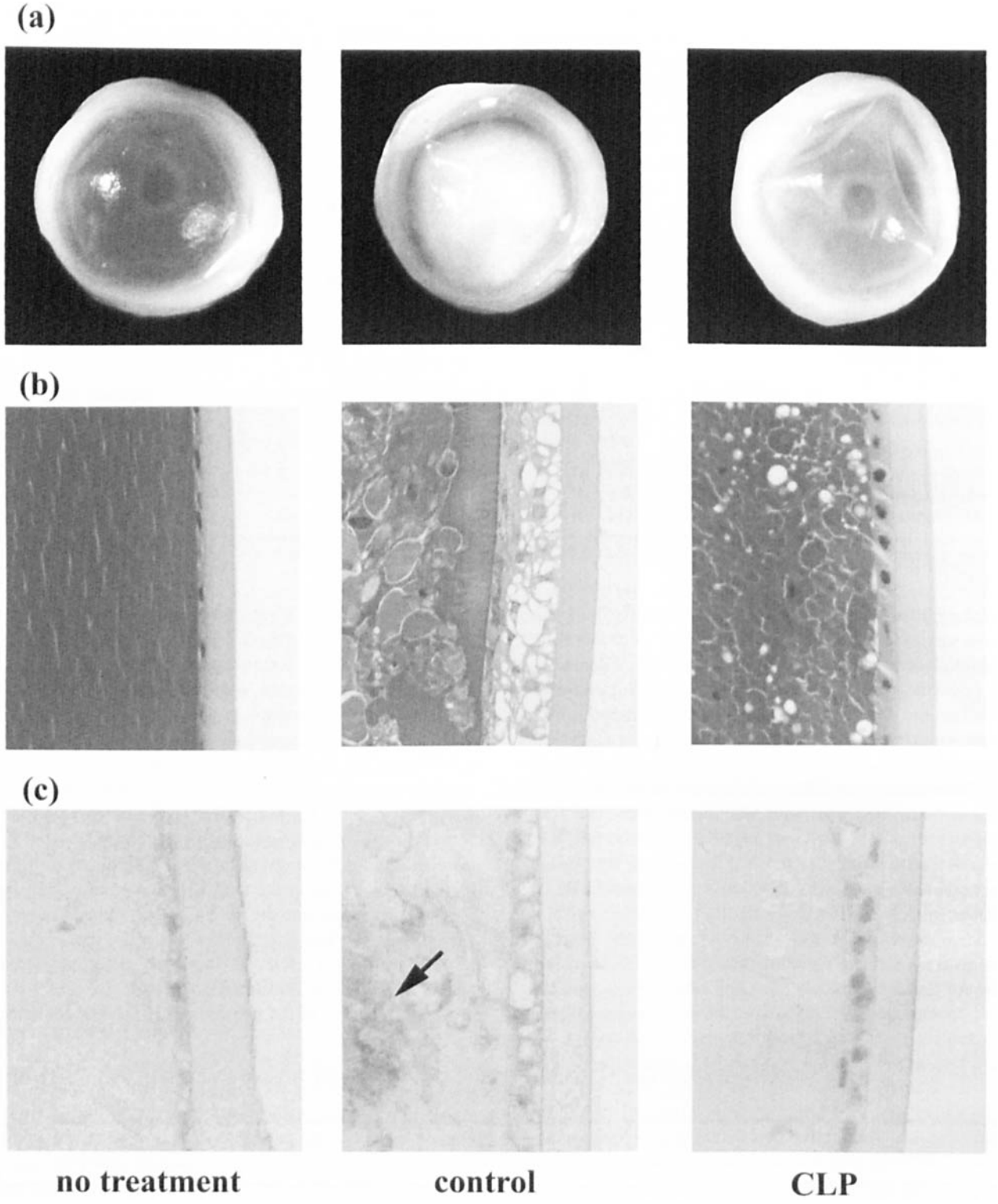

Figure 1. (a) Photograph of cataract formation. (b) Histological changes associated with cataract formation stained with hematoxylin and eosin. (c) The production of HNE in a rat lens detected by immunohistochemical staining.

in the lens of nondiabetic rats. The HNE adducts were widely distributed in the lens fibers of the STZ/normal diet group. In contrast, only slight staining was apparent in any of the eyes of the CLP-treated rats.

Discussion

In this study, rats with diabetes induced by treatment with STZ had continuously elevated levels of plasma glucose and glycosuria as described in many previous

Table 4. Cacao Proanthocyanidins Decrease Cortical Fiber Liquefaction and Focal Hyperplasia in STZ Rat Lens[a]

	Untreated control (n = 8)		STZ/normal diet (n = 9)		STZ/CLP diet (n = 8)	
Cortical fiber, liquefaction						
No observable change	16	A	0	B	0	C
Slight	0		0		0	
Mild	0		8		14	
Moderate	0		6		2	
Severe	0		4		0	
Lens, focal hyperplasia						
No observable change	16	A	6	B	10	C
Slight	0		5		5	
Mild	0		7		1	
Moderate	0		0		0	
Severe	0		0		0	

[a] Histological analyses of the lens were evaluated in 20 random areas (0.0625 mm^2 /area) at a magnification of ×400. The grading of cortical fiber liquefaction and focal hyperplasia of lenses were assessed as follows: no changes, no remarkable changes; slight, less faint changes; mild, faint changes; moderate, moderate changes; severe, evident changes. The chi-square test was used to analyze the difference in variation of between test groups. Values within a row not sharing the same letters are significantly different, $P < 0.05$.

investigations. While dietary supplementation with CLP did not affect hyperglycemia or plasma TBAR concentration, AAPH-induced production of LPO was decreased. Moreover, the formation of cataracts was observed in the STZ/normal diet animals 5 weeks after STZ treatment but not in CLP-treated animals until the eighth week (data not shown). These gross changes were confirmed histochemically by the finding that HNE, a marker of oxidative stress, was only detected when the rats were fed a normal diet. These observations suggest that the CLP extract may prevent cataract formation by decreasing oxidative stress induced by hyperglycemia.

Proanthocyanidins in cacao include monomeric (+)-catechin and (−)-epicatechin, dimeric procyanidin B2, trimeric procyanidin C1, and tetrameric cinnamtannin A2. This mixture has been shown to have potent antioxidative activity (28, 29). *In vitro* studies have demonstrated that each of these compounds scavenges superoxides and hydroxy radicals (30) and inhibits the formation of nitrotyrosine (31, 32). A recent report also indicates that catechins are more efficiently absorbed from the gastrointestinal tract than other flavonoids (33). We found that (−)-epicatechin and its metabolites reached their highest levels in plasma 1 or 2 hrs after feeding healthy volunteers cocoa or chocolate (34, 35). Recovery of (−)-epicatechin-related compounds in the urine was 26%–28%. We confirmed similar bioavailability of the (−)-epicatechin in cocoa in rats and found a correlation between antioxidative status and (−)-epicatechin concentration in plasma (36). However, in the present study, the procyanidin B2, (−)-epicatechin dimer was poorly absorbed, with only 0.5% of the procyanidin B2-related substances that were administered being recovered in the urine (37). While the bioavailability of proanthocyanidins remains debatable, many studies in both experimental animals and humans have shown that ingestion of these compounds in cocoa, wine, and apples affects physiological activities *in vivo*, such as preventing the development of atherosclerosis and some types of cancer (38–40). Although monomeric proanthocyanidins such as (−)-epicatechin and (+)-catechin have relatively high antioxidative activity compared with other compounds in CLP, further investigation is needed to identify the components that contribute to these physiological changes.

Hyperglycemia increases the production of reactive oxygen species (ROS) leading to severe oxidative stress (41, 42). Superoxide radicals are produced by glucose autooxidation in association with the formation of glycates of plasma and cell surface proteins; they also arise as a consequence of decreased cellular levels of glutathione (43–46). The imbalance in cellular redox potential may play a role in the development of diabetic complications. Cataract results not only from osmotic stress but also from oxidative stress caused by reduced cellular levels of glutathione following activation of the sorbitol pathway (47–49). Our observations with CLP concur with other reports that dietary supplementation with antioxidants such as vitamin C (50), carotenoids (51), and α-lipoic acid (52) decreases cataract formation. On the other hand, increased ROS production in mitochondrial complex II in response to elevated glucose may also reduce diabetic complications (53). This suggests that the distribution of antioxidants within the cell organelle is likely to be important.

It has been reported recently that the production of ROS and aldehyde end products such as HNE in diabetes may lead to diacylglycerol production and activation of protein kinase C and transcription factor, nuclear factor kappa B (53). These biochemical changes may contribute to the development of chronic diabetic complications. Because proanthocyanidins including catechins inhibit PKC activity (54, 55), it is possible that the preventative effects of CLP on cataract formation are the consequence of the combined action of antioxidative activity and modulation of cellular signal transduction.

The dosage of CLP used in this study is equivalent to the content of polyphenols in 0.75 kg of chocolate. Although supplementation with chocolate would seem paradoxical in diabetic patients, we have shown that a component of chocolate has value in preventing diabetic complications. The possibility of developing a low-carbohydrate chocolate food that does not cause hyperglycemia should be considered for diabetic patients. In conclusion, this study supports the possibility that proanthocyanidins in CLP reduce oxidative stress and prevent cataract formation in the STZ rat.

1. Haffner SM, Agil A, Mykkanen L, Stern MP, Jialal I. Plasma oxidizability in subjects with normal glucose tolerance, impaired glucose tolerance, and NIDDM. Diabetes Care 18:646–653, 1995.
2. Maxwell SR, Thomason H, Sandler D, LeGuen C, Baxter MA, Thorpe GH, Jones AF, Barnett AH. Poor glycaemic control is associated with reduced serum free radical scavenging (antioxidant) activity in non-insulin-dependent diabetes mellitus. Ann Clin Biochem 34:638–644, 1997.
3. Polidori MC, Stahl W, Eichler O, Niestroj I, Sies H. Profiles of antioxidants in human plasma. Free Radic Biol Med 30:456–462, 2001.
4. Cho SY, Park JY, Park EM, Choi MS, Lee MK, Jeon SM, Jang MK, Kim MJ, Park YB. Alternation of hepatic antioxidant enzyme activities and lipid profile in streptozotocin-induced diabetic rats by supplementation of dandelion water extract. Clin Chim Acta 317:109–111, 2002.
5. Matkovics B, Kotorman M, Varga IS, Hai DQ, Varga C. Oxidative stress in experimental diabetes induced by streptozotocin. Acta Physiol Hung 85:29–38, 1997.
6. Lerner BC, Varma SD, Richards RD. Polyol pathway metabolites in human cataracts. Correlation of circulating glycosylated hemoglobin content and fasting blood glucose levels. Arch Ophthalmol 102: 917–920, 1984.
7. Cogan DG, Kinoshita JH, Kador PF, Robison G, Datilis MB, Cobo LM, Kupfer C. NIH conference. Aldose reductase and complications of diabetes. Ann Intern Med 101:82–91, 1984.
8. Van Dam PS, Van Asbeck BS, Erkelens DW, Marx JJ, Gispen WH, Bravenboer B. The role of oxidative stress in neuropathy and other diabetic complications. Diabetes Metab Rev 11:181–192, 1995.
9. Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. Diabetes Care 19:257–267, 1996.
10. Wolff SP. Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. Br Med Bull 49:642–652, 1993.
11. Packer L, Kraemer K, Rimbach G. Molecular aspects of lipoic acid in the prevention of diabetes complications. Nutrition 17:888–895, 2001.
12. Sajithlal GB, Chithra P, Chandrakasan G. Effect of curcumin on the advanced glycation and cross-linking of collagen in diabetic rats. Biochem Pharmacol 56:1607–1614, 1998.
13. Agardh E, Hultberg B, Agardh C. Effects of inhibition of glycation and oxidative stress on the development of cataract and retinal vessel abnormalities in diabetic rats. Curr Eye Res 21:543–549, 2000.
14. Forsyth WGC. Cacao polyphenolic substances. Biochem J 51:511–516, 1951.
15. Porter LJ, Ma A, Chan BG. Cacao procyanidins; major flavonoids and identification of some minor metabolites. Phytochemistry 30: 1657–1663, 1991.
16. Adamson GE, Lazarus SA, Mitchell AE, Prior RL, Cao G, Jacobs PH, Kremers BG, Hammerstone JF, Rucker RB, Ritter KA, Schmitz HH. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. J Agric Food Chem 47:4184–4188, 1999.
17. Hatano T, Miyatake H, Natsume M, Osakabe N, Takizawa T, Ito H, Yoshida T. Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. Phytochemistry 59:749–758, 2002.
18. Osakabe N, Yamagishi M, Sanbongi C, Natsume M, Takizawa T, Osawa T. The antioxidative substances in cacao liquor. J Nutr Sci Vitaminol 44:313–321, 1998.
19. Hatano T, Miyatake H, Natsume M, Osakabe N, Takizawa T, Ito H, Yoshida T. Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. Phytochemistry 59:749–758, 2002.
20. Yamagishi M, Osakab N, Takizawa T, Osawa T. Cacao liquor polyphenols reduce oxidative stress without maintaining alpha-tocopherol levels in rats fed a vitamin E-deficient diet. Lipids 36:67–71, 2001.
21. Osakabe N, Natsume M, Adachi T, Yamagishi M, Hirano R, Takizawa T, Itakura H, Kondo K. Effects of cacao liquor polyphenols on the susceptibility of low-density lipoprotein to oxidation in hypercholesterolemic rabbits. J Atheroscler Thromb 7:164–168, 2000.
22. Osakabe N, Baba S, Yasuda A, Iwamoto T, Kamiyama M, Takizawa T, Itakura H, Kondo K. Daily cocoa intake reduces the susceptibility of low-density lipoprotein to oxidation as demonstrated in healthy human volunteers. Free Radic Res 34:93–99, 2001.
23. Porter LJ, Hrstich LN, Chan BG. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 1986: 223–230, 1986.
24. Natsume M, Osakabe N, Yamagishi M, Takizawa T, Nakamura T, Miyatake H, Hatano T, Yoshida T. Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal-phase and reversed-phase HPLC. Biosci Biotechnol Biochem 64:2581–2587, 2000.
25. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358, 1979.
26. Johnson RN, Metcalf PA, Baker JR. Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. Clin Chim Acta 127:87–95, 1983.
27. Lotito SB, Fraga CG. (+)-Catechin prevents human plasma oxidation. Free Radic Biol Med 24:435–441, 1998.
28. Lotito SB, Actis-Goretta L, Renart ML, Caligiuri M, Rein D, Schmitz HH, Steinberg FM, Keen CL, Fraga CG. Influence of oligomer chain length on the antioxidant activity of procyanidins. Biochem Biophys Res Commun 276:945–951, 2000.
29. Osakabe N, Yasuda A, Natsume M, Takizawa T, Terao J, Kondo K. Catechins and their oligomers linked by C4 → C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. Proc Soc Exp Biol Med 227:51–56, 2002.
30. da Silva JMR, Darmon N, Fernandez Y, Mitjavila S. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J Agric Food Chem 39:1549–1552, 1991.
31. Arteel GE, Schroeder P, Sies H. Reactions of peroxynitrite with cocoa procyanidin oligomers. J Nutr 130:2100S–2104S, 2000.
32. Arteel GE, Sies H. Protection against peroxynitrite by cocoa polyphenol oligomers. FEBS Lett 462:167–170, 1999.
33. Donovan JL, Crespy V, Manach C, Morand C, Besson C, Scalbert A, Remesy C. Catechin is metabolized by both the small intestine and liver of rats. J Nutr 131:1753–1757, 2001.
34. Baba S, Osakabe N, Yasuda A, Natsume M, Takizawa T, Nakamura T, Terao J. Bioavailability of (−)-epicatechin upon intake of chocolate and cocoa in human volunteers. Free Radic Res 33:635–641, 2000.
35. Richelle M, Tavazzi I, Enslen M, Offord EA. Plasma kinetics in man of epicatechin from black chocolate. Eur J Clin Nutr 53:22–26, 1999.
36. Baba S, Osakabe N, Natsume M, Yasuda A, Takizawa T, Nakamura T, Terao J. Cocoa powder enhances the level of antioxidative activity in rat plasma. Br J Nutr 84:673–680, 2000.
37. Baba S, Osakabe N, Natsume M, Terao J. Absorption and urinary excretion of procyanidin B2 (epicatechin-(4beta-8)-epicatechin) in rats. Free Radic Biol Med 33:142–148, 2002.
38. Cestaro B, Simonetti P, Cervato G, Brusamolino A, Gatti P, Testolin G. Red wine effects on peroxidation indexes of rat plasma and erythrocytes. Int J Food Sci Nutr 47:181–189, 1996.
39. Kondo K, Matsumoto A, Kurata H, Tanahashi H, Koda H, Amachi T, Itakura H. Inhibition of oxidation of low-density lipoprotein with red wine. Lancet 344:1152, 1994.

40. de Rijke YB, Demacker PN, Assen NA, Sloots LM, Katan MB, Stalenhoef AF. Red wine consumption and oxidation of low-density lipoproteins. Lancet 345:325–326, 1995.
41. Oberley LW. Free radicals and diabetes. Free Radic Biol Med 5: 113–124, 1988.
42. Wolff SP. Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. Br Med Bull 49:642–652, 1993.
43. Sakurai T, Tsuchiya S. Superoxide production from nonenzymatically glycated protein. FEBS Lett 236:406–410, 1988.
44. Van Dam PS, Van Asbeck BS, Erkelens DW, Marx JJ, Gispen WH, Bravenboer B. The role of oxidative stress in neuropathy and other diabetic complications. Diabetes Metab Rev 11:181–192, 1995.
45. Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes 40:405–412, 1991.
46. Aragno M, Parola S, Tamagno E, Brignardello E, Manti R, Danni O, Boccuzzi G. Oxidative derangement in rat synaptosomes induced by hyperglycaemia: restorative effect of dehydroepiandrosterone treatment. Biochem Pharmacol 60:389–395, 2000.
47. Yeh LA, Ashton MA. The increase in lipid peroxidation in diabetic rat lens can be reversed by oral sorbinil. Metabolism 39:619–622, 1990.
48. Malone JI, Lowitt S, Cook WR. Nonosmotic diabetic cataracts. Pediatr Res 27:293–296, 1990.
49. Lou MF, Dickerson JE Jr, Garadi R, York BM Jr. Glutathione depletion in the lens of galactosemic and diabetic rats. Exp Eye Res 46:517–530, 1988.
50. Yokoyama T, Sasaki H, Giblin FJ, Reddy VN. A physiological level of ascorbate inhibits galactose cataract in guinea pigs by decreasing polyol accumulation in the lens epithelium: a dehydroascorbate-linked mechanism. Exp Eye Res 58:207–218, 1994.
51. Pollack A, Oren P, Stark AH, Eisner Z, Nyska A, Madar Z. Cataract development in sand and galactosemic rats fed a natural tomato extract. J Agric Food Chem 47:5122–5126, 1999.
52. Packer L, Kraemer K, Rimbach G. Molecular aspects of lipoic acid in the prevention of diabetes complications. Nutrition 17:888–895, 2001.
53. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787–790, 2000.
54. Takahashi T, Kamimura A, Shirai A, Yokoo Y. Several selective protein kinase C inhibitors including procyanidins promote hair growth. Skin Pharmacol Appl Skin Physiol 13:133–142, 2000.
55. Nardini M, Scaccini C, Packer L, Virgili F. *In vitro* inhibition of the activity of phosphorylase kinase, protein kinase C and protein kinase A by caffeic acid and a procyanidin-rich pine bark (*Pinus marittima*) extract. Biochim Biophys Acta 1474:219–225, 2000.