Protective Effects of Lemon Flavonoids on Oxidative Stress in Diabetic Rats

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ABSTRACT: The effects of lemon flavonoids, as crude flavonoids prepared from lemon juice, were investigated in diabetic rats. The oxidative stress of eriocitrin (eriodictyol 7-*O*-β-rutinoside) and hesperidin (hesperetin 7-*O*-β-rutinoside) on streptozotocin-induced diabetic rats was investigated. Diabetic rats were given a diet which contained 0.2% crude flavonoids, 0.2% eriocitrin, and 0.2% hesperidin. After the 28-d feeding period, the concentration of the thiobarbituric acid- reactive substance in the serum, liver, and kidney of diabetic rats administered crude flavonoids, eriocitrin, and hesperidin significantly decreased as compared with that of the diabetic group. The levels of 8-hydroxydeoxyguanosine, which is exchanged from deoxyguanosine owing to oxidative stress, in the urine of diabetic rats administered eriocitrin and hesperidin significantly decreased as compared with that of the diabetic rat group. Crude flavonoids, eriocitrin, and hesperidin suppressed the oxidative stress in the diabetic rats. These results demonstrated that dietary lemon flavonoids of eriocitrin and hesperidin play a role as antioxidant *in vivo*.

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Flavonoid compounds are widespread in the plant kingdom and are present in citrus fruits. Flavonoids in citrus fruits, known as bioflavonoids or vitamin P, exhibit beneficial effects on capillary permeability and fragility. These compounds have been investigated regarding their physiological function such as anti-inflammatory, anticarcinogenic, and antitumor activities (1–3). We have isolated antioxidative flavonoid glycosides from lemon fruit and identified eriocitrin (eriodictyol 7-*O*-β-rutinoside) of the flavanone glycoside (4), and 6,8-di-*C*-β-glucosyldiosmin and 6-*C*-β-glucosyldiosmin of the *C*-glucosylflavones (5). The flavonoid content in lemon fruit was analyzed; and hesperidin (hesperetin 7-*O*-β-rutinoside), also known as vitamin P, and eriocitrin were abundantly found. Eriocitrin had stronger antioxidative activity than the other citrus flavonoid compounds and was abundantly present in lemon and lime fruits, although hesperidin is widely distributed the among citrus fruits (6).

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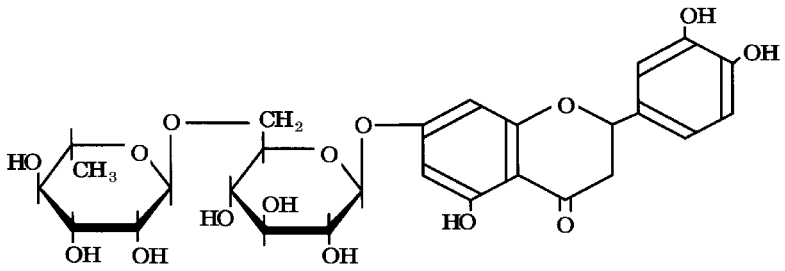
Abbreviations: CAT, catalase; CF, crude flavonoids; GSH, reduced gluthathione; GSH-Px, glutathione peroxidase; GST, glutathione *S*-transferase; 8-OHdG, 8-hydroxydeoxyguanosine; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, thiobarbituric acid-reactive substances.

Much attention has been focused on the involvement of oxidative stress of active oxygen and free radicals in aging and disease. The active oxygen species have been proposed as the attacking agents on polyunsaturated fatty acids in cell membranes. Lipid peroxidation is suspected to be strongly associated with aging and carcinogenesis (7). Antioxidants in food are expected to prevent diseases caused by oxidative stress (8,9). It is important to determine how antioxidants in food are metabolized *in vivo* and how antioxidant metabolites function *in vivo*. We had reported the metabolic pathway of eriocitrin by human intestinal bacteria for the exploration of antioxidative mechanism of eriocitrin *in vivo* (10). Oxidative modification of various proteins and lipids in plasma is found in diabetes (11). Diabetes mellitus is associated with increased lipid peroxidation, which may contribute to long-term tissue damage and increased oxidative stress as assessed by plasma hydroperoxides. Oxidative stress is an early state in the disease pathology and may contribute to the development of complications (12). Investigation of the pathophysiology of the secondary complications of diabetes is increasingly focusing on the role of oxidative stress in its initiation and progression (13). There is now ample evidence supporting the involvement of reactive oxygen species in this disease.

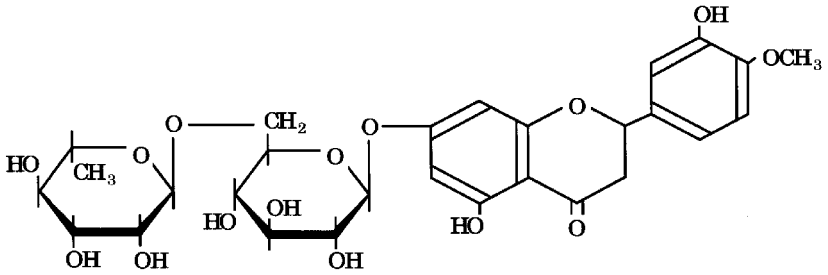
In this study, we examined whether dietary lemon flavonoids suppress oxidative stress *in vivo*, and determined whether these compounds are useful in the prevention of diabetic complications caused by oxidative stress. Oxidative stress was induced in rats using streptozocin (STZ), which is toxic to β-cells and is widely used for the induction of experimental diabetes mellitus (14). The protective effects of the lemon flavonoids eriocitrin and hesperidin, as well as crude flavonoids (CF) prepared from lemon juice, on oxidative stress were then determined.

EXPERIMENTAL PROCEDURES

Materials and chemicals. CF were prepared from lemon juice (4). This was applied to a Cosmosil 75C 18-OPN ODS column (Nacalai Tesque, Inc., Kyoto, Japan), which was washed with water and successively eluted with 100% methanol. The eluate was concentrated under reduced pressure and lyophilized. The lyophilized powder containing CF was examined for flavonoid glycosides content by high-performance



eriocitrin (eriodictyol 7-*O*-β-rutinoside)



hesperidin (hesperetin 7-*O*-β-rutinoside)

SCHEME 1

liquid chromatography according to the method of Miyake *et al.* (6). The CF (1.00 g) contained eriocitrin (385 mg), hesperidin (182 mg), 6,8-di-*C*-β-glucosyldiosmin (72.2 mg), narirutin (25.0 mg), and diosmin (22.0 mg). Eriocitrin was prepared from lemon peel extract (4), and the purity was greater than 99.0% as determined by high-performance liquid chromatography. Hesperidin was obtained as a reagent-grade chemical from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The chemical structures of eriocitrin and hesperidin are shown in Scheme 1.

Animals and diets. Five-week-old male Wistar strain rats (Japan SLC, Ltd., Hamamatsu, Japan) were individually housed in stainless-steel cages with screen bottoms. The animals were kept under controlled conditions with a 12-h light/dark cycle (0800–2000 light) and at 22–24°C. All rats were fed commercial CE-2 pellets (Crea Japan, Ltd., Tokyo, Japan) and water *ad libitum*. Diabetes was induced in ether-anesthetized rats by the administration of STZ (60 mg/kg) as a freshly prepared solution (50 mg/mL) in saline *via* intraperitoneal injection. Diabetic rats were fed on CE-2 pellets and water *ad libitum* for 48 h after the administration of STZ. The increase of glucose content in urine of diabetic rats was checked by a commercial glucose kit (TES-Tape, Shionogi Pharmaceutical Company, Ltd., Tokyo, Japan). Three additional groups of diabetic rats were fed water *ad libitum* and CE-2 pellets containing CF, eriocitrin, or hesperidin for 28 d.

This experiment was carried out with five groups of five rats. The control group was the nondiabetic rat group (C), which had not received STZ. Diabetic rats were randomly assigned to four groups of five animals: a diabetic group fed CE-2 pellets (D); a diabetic group fed CE-2 pellets containing 0.2% CF (S+CF); a diabetic group fed CE-2 pellets containing 0.2% eriocitrin (D+ERI); and a diabetic group fed CE-2 pellets containing 0.2% hesperidin (D+HES). The feeds were prepared by mixing CE-2 pellets with the powder of CF, eriocitrin, and hesperidin. The food intake (g) of individual rats was measured daily. Body weight of the rats was measured weekly.

Tissue collection and processing. After 27 d on diet, rats were placed in individual metabolic cages in order to collect urine for 24 h. The collected urine was used to determine 8-hydroxydeoxyguanosine (8-OHdG).

After feeding the rats for 27 d, they were fasted for 10 h. On day 28 the rats were then anesthetized with ether, blood was collected into tubes by cardiac puncture, and the livers and kidneys were removed and stored at −80°C. Liver and kidney tissues were homogenized twice in 10 vol of 50 mM sodium phosphate buffer (pH 7.4) at 4°C for 15 s. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 1,900 × *g* for 5 min. The supernatant was used for various measurements. Blood was centrifuged at 1,900 × *g* for 10 min to obtain serum.

The serum, the supernatant of homogenized liver and kidney, and the urine of rats were stored at −80°C and processed for biochemical measurements within 2 wk.

Measurement of thiobarbituric acid-reactive substances (TBARS). The serum TBARS concentration was determined by the method of Yagi (15) and is expressed as nmol of malondialdehyde per mL of blood. The liver and kidney TBARS concentrations were measured by the method of Uchiyama

and Mihara (16) using a homogenate prepared by homogenizing 1 g of frozen rat liver and kidney (a section from the main lobe) with 9 mL of 1.15% KCl.

Determination of 8-OHdG. Rat urine was centrifuged at 1,900 × *g* and the precipitate was removed. The content of 8-OHdG in the urine was measured using the 8-OHdG enzyme-linked immunosorbent assay kit (Japan Institute for Control Aging, Fukuroi City, Japan) (17,18). Creatinine content in the urine was measured using commercial kits (Wako Pure Chemical Industries, Ltd.) (19).

Measurement of antioxidative enzyme activities and glutathione. The supernatants of homogenized liver and kidney were analyzed for their antioxidant enzyme activities and content of glutathione. Superoxide dismutase (SOD) activity was measured by the xanthine/xanthine oxidase/nitro-blue tetrazolium system (20), and the catalase (CAT) activity was measured by a spectrophotometric method, following a decrease in absorbance at 240 nm and 25°C, due to hydrogen peroxide decomposition (21). Glutathione peroxidase (GSH-Px) activity was measured using the procedures of Lawrence and Burk (22) with *t*-butyl hydroperoxide as the substrate, following the decrease in absorbance of NADPH at 340 nm. Glutathione *S*-transferase (GST) activity was assayed by measuring the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (23). The content of GSH was measured by an enzymatic method (24). Protein content was measured using a commercial protein assay kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

Lipid analyses. Total cholesterol and triglyceride in the serum were enzymatically measured using commercial kits (cholesterol E-test and triglyceride E-test, respectively; Wako Pure Chemical Industries, Ltd.).

Statistical analyses. All values were expressed as mean ± S.E. ($n = 5$). The data for each of the five groups were statistically analyzed by Duncan's multiple-range test, and significant differences in the means were $P < 0.05$.

RESULTS AND DISCUSSION

General features of diabetic animals. As shown in Figure 1, the growth rate of the STZ-induced diabetic rat group (D) was slower than that of the nondiabetic rat group (C), as indicated by the smaller body weight increase during the 28-d period. The growth curves of the diabetic rat groups administered lemon flavonoids as CF, eriocitrin, and hesperidin (D+CF, D+ERI, and D+HES) also showed a slower growth rate than that of the nondiabetic rat group (C). The average food intake of the diabetic rat groups (D, D+CF, D+ERI, and D+HES) in 1 d was greater than that of the nondiabetic rat group (C). As shown in Table 1, the volume of urine and the glucose level in serum and urine of the diabetic rat groups (D, D+CF, D+ERI, and D+HES) were higher than those for the nondiabetic rat group (C). The decrease in body weight gain, the increase in food intake, the high volume of urine, and the high glucose levels in blood and urine for the STZ-induced diabetic rats are typical symptoms of diabetes (25). There was no improvement in these symptoms for the diabetic rats after the administration of lemon flavonoids such as the CF, eriocitrin, and hesperidin.

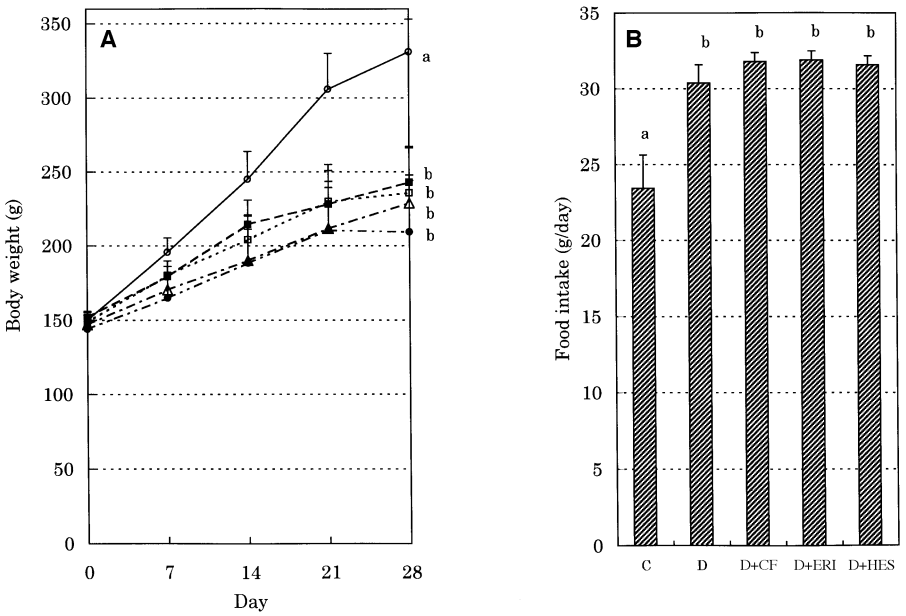


FIG. 1. Effect of dietary lemon flavonoids on body weight gain (A) and food intake (B) of diabetic rats. Diet groups (A) were: nondiabetic group (○; C), diabetic group (●, D), diabetic group administered crude flavonoids (△; D+CF), diabetic group administered eriocitrin (□; D+ERI), diabetic group administered hesperidin (■, D+HES). Values are means ± SEM of five rats per group. Lines or bars that do not share a common lowercase letter are significantly different at $P < 0.05$.

TABLE 1
Effect of Lemon Flavonoids on Liver, Kidney, Serum, and Urine of Diabetic Rats[a]

	Rat groups				
	C	D	D+CF	D+ERI	D+HES
Liver weight (% body weight)	3.95 ± 0.18[a]	5.30 ± 0.50[b]	5.42 ± 0.18[b]	5.19 ± 0.33[b]	5.08 ± 0.47[b]
Kidney weight (% body weight)	0.68 ± 0.02[a]	1.29 ± 0.13[b]	1.16 ± 0.05[b,c]	1.09 ± 0.03[c]	1.14 ± 0.10[b,c]
In serum (mL/100 mL)					
Glucose	170.6 ± 10.8[a]	532.9 ± 60.5[b]	609.3 ± 75.4[b]	521.1 ± 46.2[b]	559.7 ± 75.4[b]
Total cholesterol	42.6 ± 5.29[a]	81.8 ± 25.7[b]	77.6 ± 14.3[b]	74.8 ± 7.03[b]	79.7 ± 15.5[b]
Triglyceride	36.1 ± 3.96[a]	247 ± 77.3[b]	202 ± 45.7[b]	142 ± 71.7[b]	143 ± 34.7[b]
In urine					
Glucose (mL/100 mL)	0.07 ± 0.03[a]	10.92 ± 1.40[b]	9.81 ± 1.64[b]	11.09 ± 1.37[b]	9.47 ± 1.07[b]
Volume (mL/24 h)	10.6 ± 2.4[a]	136.7 ± 17.5[b]	151.0 ± 24.3[b]	114.3 ± 57.3[b]	115.0 ± 20.6[b]

[a]Values are means ± SEM of five rats per group. Values within the same row that do not share a common superscript roman letter are significantly different at $P < 0.05$. Abbreviations: C, nondiabetic group; D, diabetic group; D+CF, diabetic group administered crude flavonoids, (CF); D+ERI, diabetic group administered eriocitrin; D+HES, diabetic group administered hesperidin.

The liver and kidney weights (% body weight) for the diabetic rat group (D) were higher than those of the nondiabetic rat group (C) because of the hypertrophy of the liver and kidney caused by the STZ induction (Table 1). These are typical symptoms of diabetic rats (25). The kidney weights for the diabetic rat groups administered CF (D+CF) and hesperidin (D+HES) were not different from those of the diabetic rat group (D). However, kidney weights for rats fed eriocitrin (D+ERI) were significantly lower than for the diabetic rat group (D) ($P < 0.05$). The eriocitrin of lemon flavonoids may play an important role for improvement of the hypertrophy of the kidney in the diabetic rats. With respect to the liver weights, there were no differences among the diabetic rat groups. The levels of triglyceride and total cholesterol in the serum of the diabetic rats had been reported to be greatly elevated (25). The level of triglyceride in the serum of the diabetic rat groups administered lemon flavonoids (D+ERI and D+HES) was lower than in the diabetic rat group (D) but there was no significant difference ($P < 0.05$). Hesperidin decreases the triglyceride in the blood of rats fed fat (26). Eriocitrin and hesperidin may suppress the triglyceride in the blood of diabetic rats. There was no difference among the diabetic rat groups in the level of total cholesterol in serum.

TBARS levels of liver, kidney, and serum. The changes in the TBARS of the liver, kidney, and serum of diabetic rats after the administration of the lemon flavonoids are summarized in Figure 2. There was a significant increase in the TBARS of the liver, kidney, and serum in the diabetic rat group (D) compared to the control group. However, the TBARS levels of the liver, kidney, and serum in the diabetic rat groups administered lemon flavonoids (D+CF, D+ERI, and D+HES) were significantly decreased compared to the TBARS of the diabetic rat group (D). The increase of malondialdehyde, caused by lipid peroxidation, in tissue and blood of STZ-induced diabetic rats has been previously reported (13,27). The increased levels of TBARS, the reactive substance of malondialdehyde and TBA, resulted in increased levels of oxygen free radicals which attacked the polyunsaturated fatty acids in cell membranes and caused lipid peroxidation. STZ can also give rise to oxygen free radicals because of the increase in blood glucose levels in diabetes (14). An amino-carbonyl reaction, the so-called Maillard reaction, occurs *in vivo* as well as *in vitro* and is associated with the chronic complications of diabetes mellitus and aging in human beings (28). In particular, long-lived proteins such as lens crystallins, collagens, and hemoglobin may react with reducing sugars thus undergoing dehydration, rearrangement, cleavage, and polymerization reactions to produce advanced glycation end products (13,28). Lemon flavonoids of the CF, eriocitrin, and hesperidin affected the suppression of lipid oxidation in the liver, kidney, and serum. We postulated that these flavonoids play a role as free radical scavengers *in vivo* and prevent the development of complications associated with diabetes.

Determination of 8-OHdG in rat urine. Lipid peroxidation may play an important role in carcinogenesis, and there is speculation that oxidative damage can occur in DNA during the peroxidative breakdown of the membranes' polyunsaturated fatty acids (8). It is reported that mutation in mitochondrial DNA is caused by oxygen radicals and hydroxy radicals that oxidize 2′-deoxyguanosine to 8-OHdG (29). In STZ-induced diabetic rats, STZ stimulated H_2O_2 generation and caused DNA fragmentation (30). Since 8-OHdG has continued to serve as a good biomarker for the estimation of oxidative damage in DNA, we determined 8-OHdG in the urine of diabetic rats using the monoclonal antibody against 8-OHdG (17,18). The levels of total 8-OHdG over 24 h in the urine of diabetic rats and of 8-OHdG relative to creatinine content in the urine are shown in Figure 3. There was a significant increase of 8-OHdG in the urine of the diabetic rat group (D) compared to the control group. However, the level of 8-OHdG in the urine of the diabetic rat groups administered eriocitrin (D+ERI) and hesperidin (D+HES) significantly decreased compared to 8-OHdG of the diabetic rat group (D). The diabetic rat group administered the lemon CF (D+CF) did not have a significant decrease, but showed a tendency to decrease. The effects on total 8-OHdG for 24 h and 8-OHdG relative to creatinine content were almost the same. CF, eriocitrin, and hesperidin suppressed the generation 8-OHdG in the urine of the diabetic rats. This result suggests that the di-

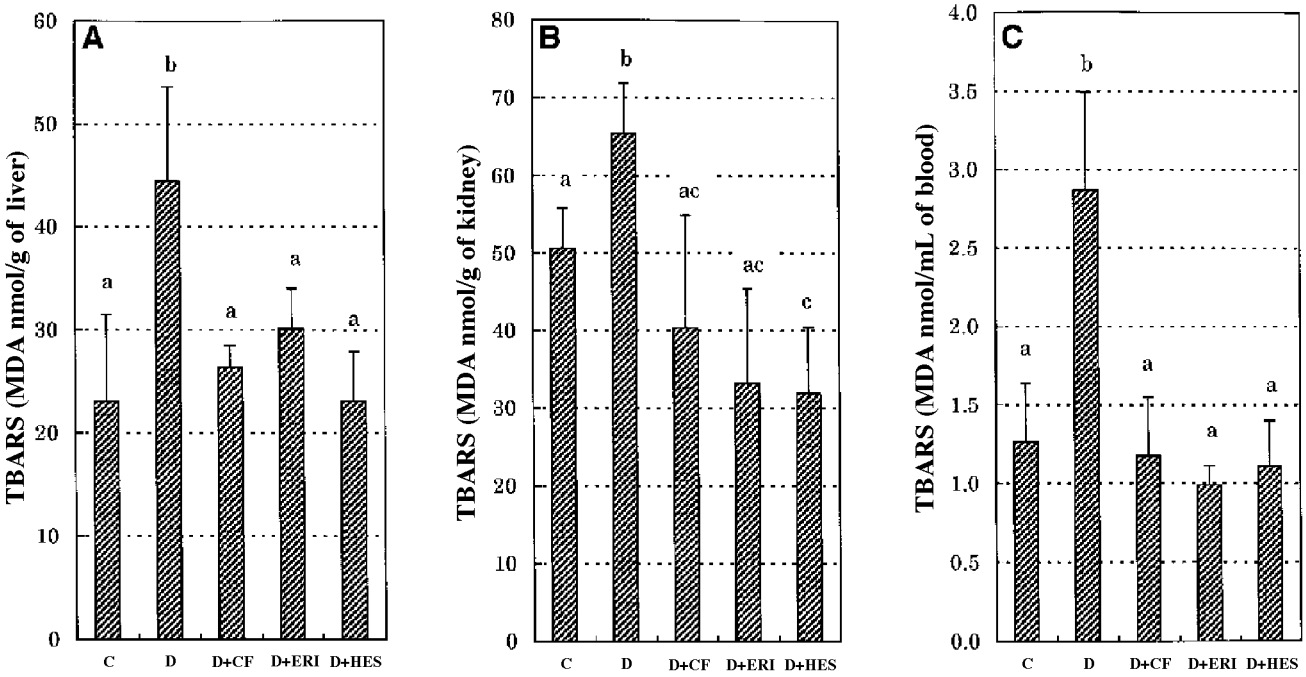


FIG. 2. Effect of lemon flavonoids on liver (A), kidney (B), and serum (C) thiobarbituric acid-reactive substances (TBARS) of diabetic rats, measured in terms of malondialdehyde. Values are means ±SEM of five rats per group. Values within the same panel that do not share a common lowercase letter are significantly different at $P < 0.05$. MDA, malondialdehyde; for abbreviations see Figure 2.

etary lemon flavonoids eriocitrin and hesperidin play a role in scavenging the free radicals generated by oxidative stress *in vivo*.

Influence of antioxidative enzyme activities. A considerable body of clinical and experimental evidence suggests the involvement of free radical-mediated oxidative processes in the pathogenesis of diabetic complications (13). The increase in the production of free radicals can result from the hyperglycemia-induced enhancement in glucose autoxidation, pro-

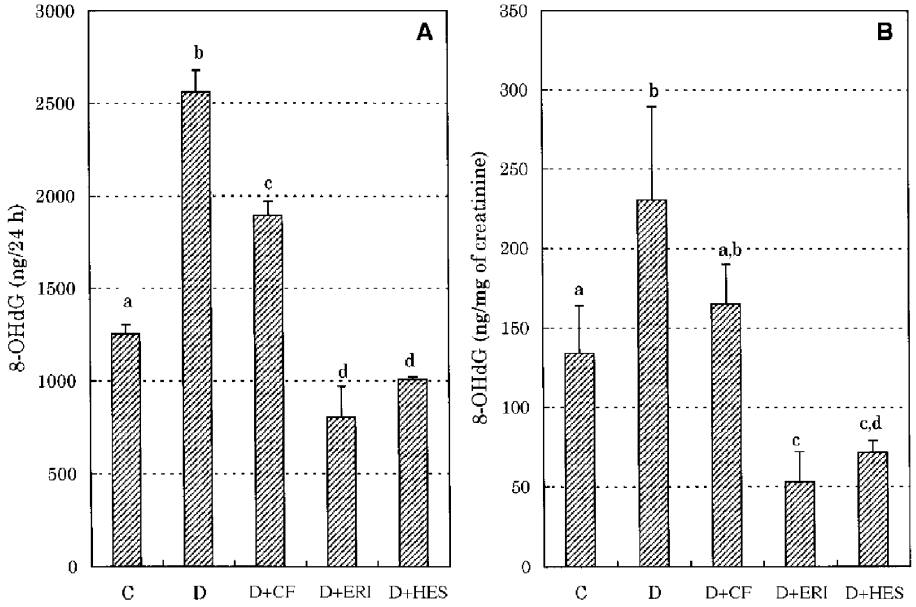


FIG. 3. Effect of lemon flavonoids on the total 8-hydroxydeoxyguanosine (8-OHdG) levels in a 24-h period (ng/24 h) (A) and on the content of 8-OHdG relative to the content of creatinine (ng/mg of creatinine) (B) in the urine of diabetic rats. Values are means ± SEM of five rats per group. Values within the same panel that do not share a common lowercase letter are significantly different at $P < 0.05$. For other abbreviations see Figure 1.

TABLE 2
Effect of Lemon Flavonoids on the Antioxidative Enzyme Activities (U/mg protein) of Diabetic Rats[a]

	Rat groups				
	C	D	D+CF	D+ERI	D+HES
Activity in liver					
GSH-Px	111.6 ± 12.2[a]	89.2 ± 5.8[b]	141.2 ± 10.3[c]	155.6 ± 10.9[c]	142.0 ± 19.1
GST	9.88 ± 1.15[a]	8.75 ± 0.93[a]	9.93 ± 1.27[a]	9.09 ± 1.33[a]	8.71 ± 0.94[a]
SOD	1.29 ± 0.11[a]	1.18 ± 0.02[a]	1.13 ± 0.10[a]	1.17 ± 0.16[a]	1.22 ± 0.15[a]
CAT	331 ± 14.1[a]	226 ± 19.4[b]	319 ± 23.9[ac]	355 ± 36.8[a]	264 ± 44.8[b,c]
Activity in kidney					
GSH-Px	87.4 ± 23.6[a]	93.0 ± 19.5[a]	137.6 ± 11.2[b]	132.2 ± 17.2[b]	133.8 ± 17.2[b]
GST	2.02 ± 0.11[a]	2.88 ± 0.29[b,c]	2.97 ± 0.28[b]	2.50 ± 0.19[c]	2.48 ± 0.40[a,b,c]
SOD	2.17 ± 0.09[a]	2.24 ± 0.10[a]	2.21 ± 0.20[a]	2.06 ± 0.14[a]	2.12 ± 0.09[a]
CAT	109 ± 17.0[a]	119 ± 20.3[a]	123 ± 24.3[a]	114 ± 23.4[a]	100 ± 25.5[a]
Content of GSH in liver[b]	7.51 ± 0.62[a]	4.89 ± 1.42[b]	15.63 ± 5.64[c]	15.56 ± 3.66[c]	11.51 ± 3.61[c]

[a]Values are means ± SEM of five rats per group. Values within the same row that do not share a common superscript roman letter are significantly different at $P < 0.05$. CAT, catalase; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GST, glutathione *S*-transferase; SOD, superoxide dismutase. For other abbreviations see Table 1.
[b]mM/mg of protein.

tein glycation, and subsequent oxidative degradation of glycated protein (28). The glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species (31). It consists of GSH and an array of functionally related enzymes. If the diabetic state is associated with a generalized increase in tissue oxidative stress, it may be reflected in changes of the tissue glutathione antioxidant system. The activities of the antioxidative enzymes SOD, CAT, GST, and GSH-Px in diabetic rats fed CF, eriocitrin, and hesperidin are shown in Table 2. The activities of SOD and GST in the liver and that of SOD and CAT in the kidney were not different among the rat groups. The activity of GSH-Px in the liver and kidney and of GST in kidney for the diabetic rat groups administered lemon flavonoids (D+CF, D+ERI, and D+HES) increased when compared with the non-diabetic rat group (C) and the diabetic rat group (D). GSH-Px and GST work together with GSH during the decomposition of hydrogen peroxide or other organic hydroperoxides (31). The lemon flavonoids eriocitrin and hesperidin may function to increase the concentration of these enzymes as well as the antioxidative activity *in vivo*. The activities of CAT and the content of GSH in the liver for the diabetic rat group (D) decreased when compared with the nondiabetic rat group (C). However, the activities for the diabetic rat groups administered lemon flavonoids (D+CF, D+ERI, and D+HES) were higher than for the diabetic rats (D). The content of GSH consumed by oxidative stress in the diabetic rats suggests that administration of the lemon flavonoids afforded an improvement.

CF in lemon fruit contained many compounds such as flavonoids and phenolic compounds. Eriocitrin and hesperidin, present in CF of lemon, had a strong suppressing effect on oxidative stress as evidenced by decreased levels of TBARS and 8-OHdG in tissues of diabetic rats. These results suggest that dietary lemon flavonoids eriocitrin and hesperidin may play a role in preventing the development of diabetes and be useful in preventing diseases caused by oxidative stress.

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