

Dietary Flavonols Protect Diabetic Human Lymphocytes Against Oxidative Damage to DNA

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Diabetic patients have reduced antioxidant defenses and suffer from an increased risk of free radical-mediated diseases such as coronary heart disease. Epidemiological evidence has suggested that antioxidant dietary flavonoids may protect against heart disease, but a biological effect has yet to be demonstrated directly in humans. In this study, 10 stable type 2 diabetic patients were treated for 2 weeks on a low-flavonol diet and for 2 weeks on the same diet supplemented with 76–110 mg of flavonols (mostly quercetin) provided by 400 g of onions (and tomato sauce) and six cups of tea daily. Freshly collected lymphocytes were subjected to standard oxidative challenge with hydrogen peroxide, and DNA damage was measured by single-cell gel electrophoresis. Fasting plasma flavonol concentrations (measured by high-performance liquid chromatography) were 5.6 ± 2.9 ng/ml on the low-flavonol diet and increased 12-fold to 72.1 ± 15.8 ng/ml on the high-flavonol diet ($P < 0.001$). Oxidative damage to lymphocyte DNA was 220 ± 12 on an arbitrary scale of 0–400 U on the low-flavonol diet and 192 ± 14 on the high-flavonol diet ($P = 0.037$). This decrease was not accounted for by any change in the measurements of diabetic control (fasting plasma glucose or fructosamine) or by any change in the plasma levels of known antioxidants, including vitamin C, carotenoids, α -tocopherol, urate, albumin, and bilirubin. In conclusion, we have shown a biological effect of potential medical importance that appears to be associated with the absorption of dietary flavonols. *Diabetes* 48:176–181, 1999

Diabetic patients, both type 1 and 2, exhibit abnormal antioxidant status, auto-oxidation of glucose, and excess glycosylated proteins (1–4). Oxidative stress in diabetes leads to tissue damage, with lipid peroxidation, inactivation of proteins, and protein glycation as intermediate mechanisms (5) for complications including retinopathy, nephropathy, and coronary heart disease (6–9). Dietary antioxidant compounds, including ascorbic

acid and tocopherol, offer some protection against these complications through their roles as inhibitors of glycation and as free radical scavengers (10,11). The synthetic flavonoid diosmin inhibits nonenzymatic protein glycation (12).

Flavonoids are antioxidant polyphenolic compounds ubiquitously found in plants, typically as sugar conjugates of six subgroups; flavonols, flavones, flavanones, isoflavones, anthocyanins, and catechins. They are present in significant amounts in commonly consumed fruits and vegetables, particularly onions, apples, and tomatoes, and in beverages such as red wine and tea. Consumption of flavonoids, particularly the flavonol quercetin (3,5,7,3',4'-pentahydroxy-flavone), has been associated with a reduced incidence of heart disease and cancer (13–15), hypothesized to be due to their antioxidant properties. We have recently shown that flavonoids have very high antioxidant activities as compared with vitamin C, with quercetin and its conjugates consistently among the most potent (M.N., H. Millar, N.S., M.E.J.L., unpublished observations). Although in vitro and epidemiological evidence indicate an important dietary role for flavonoids (15,17,18), flavonol absorption has been disputed. Current evidence suggests that while quercetin is poorly absorbed, its conjugates have been detected in plasma (19).

The present study was designed to establish, first, whether dietary supplements of flavonol-rich foods were absorbed consistently, and second, whether they might have a biological effect in the protection against oxidative stress in type 2 diabetic patients. High-performance liquid chromatography (HPLC) analysis was used to determine the extent of flavonol absorption, and a single-cell gel electrophoresis (SCGE) assay was used to determine the level of antioxidant defenses by measuring the oxidative damage from hydrogen peroxide incurred by fresh lymphocytes after both the low-flavonol and supplemented-flavonol diets. Possible confounding effects from other antioxidant systems were excluded by the measurement of known antioxidant vitamins, α -tocopherol, carotenoids, and other compounds, such as urate and albumin.

RESEARCH DESIGN AND METHODS

Subjects and study design. Patients with stable type 2 diabetes, who were healthy in other respects, were recruited from outpatient clinics. The inclusion criteria consisted of the following: no medication change during the study period, no vitamin supplements, and not pregnant. Prestudy diets (4-day weighed inventory) were analyzed using Compeat (Table 1). Of the 10 subjects, 4 were treated with diet and oral hypoglycemic agents (2 with sulfonylureas, 2 with biguanide) and 6 by diet alone. They were assigned, in random order, to follow either a high- (supplemented) or low-flavonol diet for 14-day periods in a crossover study. Two high-flavonol diets were used, prepared as a palatable dish to be eaten in three equal portions with meals. Five subjects received a simple fried onion supplement (60.2 mg flavonols/day), and five sub-

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HPLC, high-performance liquid chromatography; IGT, impaired glucose tolerance; SCGE, single-cell gel electrophoresis; TEAC, Trolox equivalent antioxidant capacity.

jects received the same onion supplement with tomato ketchup and herbs (93.7 mg flavonols/day). All subjects also received a daily tea supplement containing 16.7 mg of flavonols. Total flavonol supplements were thus 76.3 and 110.4 mg daily (Table 2). Fasting blood samples and 24-h urine collections were obtained at baseline and for low- and high-flavonol diets.

The protocol was approved by the Glasgow Royal Infirmary Medical Research Ethical Committee, and all subjects signed a form of informed consent.

Dietary intervention

Low-flavonol diet (28 days). Advice was provided by a state-registered dietician to avoid eating foods high in flavonols, including certain drinks (red wine, fruit juices of all varieties, tea), fruits (especially apples, oranges, strawberries, grapes, berries, currants, and sultanas), vegetables (particularly onion, garlic, shallots, tomatoes, lettuce, celery, beans, parsley, cabbage leaves, and peppers [red, green, yellow]), and nuts (walnut, hazelnut, peanut). Advice was given to maintain normal energy intake in order to avoid weight change.

High-flavonol diet. The high-flavonol diet involved 14 days of supplementation to the low-flavonol diet with either an onion supplement or an onion, tomato ketchup, and herb supplement.

Onion supplement. Yellow English onions (400 g), without dry skin, were chopped into medium slices and fried lightly for 1.5 min with 20 g olive oil (extra virgin) and consumed in three equal mealtime supplements.

Onion, tomato ketchup, and herb supplement. Yellow English onions (400 g), without dry skin, were chopped into medium slices and lightly fried for 1.5 min with 20 g olive oil (extra virgin), 20 g tomato ketchup (Heinz), and 1 g Italian seasoning herbs (Safeway) and consumed in three equal mealtime supplements. All subjects also received six mugs (250 ml) of tea per day (Typhoo tea bag, 5 min infusion).

Flavonols in supplements were measured by HPLC (Table 2). The onion and tomato ketchup supplements were analyzed for their antioxidant vitamin contents. Vitamin C content was measured using a colorimetric method and was 3.6 mg/100 g, while vitamins E and A were both undetectable (<2 mg/100 g) using HPLC methods. Tea did not contain any of these vitamins.

Measurements of antioxidant defense. The SCGE or "Comet" assay is based on the principle that low concentrations of hydrogen peroxide cause oxidative damage to lymphocyte DNA. Under alkaline conditions, DNA loops containing breaks unwind and are released from the nucleus, forming a "comet tail" after gel electrophoresis. The size of the comet tail reflects the extent of DNA strand breaks, and can be quantified by image intensification and computer analysis or by visual grading. Pretreatment of lymphocytes with antioxidant compounds decreases H₂O₂-induced DNA damage, and this forms the basis for using the assay to assess antioxidant defenses.

The full method has been described elsewhere (20). Briefly, fresh peripheral human lymphocytes were isolated by centrifugation with Histopaque 1077 at 40°C, washed with phosphate-buffered saline, and treated with H₂O₂ (100 µmol/l, 5 min on ice), then suspended in agarose (Gibco, Paisley, Scotland, U.K.) and lysed with 1% Triton X-100 for 1 h. Gel electrophoresis at pH 13 for 30 min at 4°C was then used to estimate DNA breaks of 300 lysed nuclei (comets whose tails contain uncoiled DNA) in each experiment at each concentration. Visual scoring was used routinely, validated using an Imaging Research BRS2 Image Analyser (Imaging Research, Ontario, Canada) with the fluorescent dye ethidium bromide to quantify comet-tail DNA. The percentage of DNA in the tail at different visual grades of damage fell in the following nonoverlapping ranges: grade 0 (no damage) <5%, grade 1 (low damage) 5–25%, grade 2 (medium damage) 25–45%, grade 3 (high damage) 45–70%, and grade 4 (very high damage) >70%. A total damage score for the slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and summing over all grades (giving a maximum possible score of 400, corresponding to 100 cells of grade 4). In each experiment, duplicate slides were prepared and scored.

The endonuclease III assay, an adaptation of the SCGE assay, was used to attempt to quantify the presence of oxidative DNA base damage. Endonuclease III specifically nicks DNA at the sites of oxidized pyrimidines, thereby converting oxidized bases to strand breaks, which are detected by SCGE as above. Endonuclease III, purified by the method of Asahara et al. (21) as modified by Collins et al. (22), was provided as a gift by Dr. A. Collins, Rowett Research Institute, Aberdeen. After lysis, lymphocyte slides were treated with 50 µl of endonuclease III in buffer (1 µg of protein/ml) and incubated for 30 min at 37°C (23). Alkaline treatment, electrophoresis, neutralizing, staining, quantification of DNA damage, and slide scoring followed as in the SCGE assay.

Trolox equivalent antioxidant capacity (TEAC) was used to estimate antioxidant capacity of plasma, using a commercial kit (Randox total antioxidant status kit; Randox Laboratories, Crumlin, Northern Ireland, U.K.) (24).

Biochemical measurements. Concentrations of free and conjugated flavonols in plasma, urine, and food (test meals) were determined by reversed-phase HPLC. Briefly, samples were hydrolyzed in 1.2 mol/l HCl and 50% methanol at 90°C for 2–3 h in the presence of the antioxidant diethyldithiocarbamic acid. Samples were then analyzed using a Shimadzu (Kyoto, Japan) LC-10A series automated

liquid chromatograph. Derivatized flavonol complexes were detected with the RF-10AXL fluorimeter (Roche Diagnostics, Lewes, England, U.K.) (excitation 425 nm, emission 480 nm). The limit of flavonol detection was <1 ng, and linear 5–250 ng calibration curves were obtained for myricetin, quercetin, isorhamnetin, and kaempferol (25). Free flavonols were detected in the unhydrolyzed samples, with acid hydrolysis cleaving the flavonol conjugates and releasing the aglycone. The conjugated flavonol level was estimated by subtracting the amount of free flavonol detected in the unhydrolyzed sample from the quantity present in the acid-hydrolyzed extract.

Routine biochemistry was measured using standard laboratory methods, and standard HPLC methods were used for retinol, tocopherol, and carotenoids (26). Ascorbic acid was measured by a spectrophotometric method (27). GSH-Px activity was determined using a commercial kit using a coupled enzyme procedure with sensitivity to 1 U/g (Cobas Mira; Roche, Lewes, England, U.K.). Superoxide dismutase activity was determined using an assay kit from Calbiochem (Nottingham, U.K.). Plasma selenium was measured by electrothermal atomic absorption spectrometry with deuterium-arc background correction. The detection limit was 6 µg/l in the original sample (28).

Statistical analysis. Appropriate parametric or nonparametric analyses for paired data were used to compare data at the ends of low- and high-flavonol diet periods. Wilcoxon's test was used for all comparisons except SCGE and endonuclease III, whose data appeared to be normally distributed, and thus paired Student's *t* tests were used. All data are presented as means ± SE. A *P* value <0.05 is regarded as statistically significant.

RESULTS

Subjects reported high compliance with the low-flavonol background diet throughout the study. The dietary supplements of onions and tea were well accepted and tolerated. Body weights did not change during the study (baseline 81.2 ± 3.5 kg, high-flavonol 81.5 ± 4 kg). Three subjects were smokers and did not change their habit during the study.

On the low-flavonol diet, plasma flavonols were detectable in fasting plasma (above 1 ng/ml) in three subjects (mean 18.6 ng/ml) and undetectable in seven subjects. The mean concentration for the whole group was 5.6 ± 2.9 ng/ml. On the high-flavonol diets, fasting plasma flavonols were detectable in all subjects, with a mean concentration of 72.1 ± 15.6 ng/ml

TABLE 1

Characteristics of diabetic patients (five men, five women) and background daily nutrient intake assessed by a 4-day weighed diet diary (2 weekdays, 2 weekend days)

Age (years)	60.1 ± 7 (50–74)
Height (m)	1.64 ± 0.1 (1.49–1.83)
Weight (kg)	81.2 ± 11.1 (69.4–107.2)
BMI (kg/m ²)	30.2 ± 3.5 (24.9–38.3)
Duration of diabetes (years)	6 ± 4 (2–11)
Nutrient intake	
Energy (kcal)	1,990 ± 703 (805–2,897)
Fat (%E)	38.4 ± 6.2 (32.3–53.3)
Protein (%E)	19.9 ± 3.3 (16.8–27.7)
Carbohydrate (%E)	39.0 ± 7.3 (28.5–49.5)
Ethanol (%E)	2.5 ± 4.0 (0.0–10.5)
Nonstarch polysaccharides (g/day)	14.6 ± 8.4 (3.0–32.0)
Iron (mg/day)	16.0 ± 7.2 (4.8–31.3)
Copper (mg/day)	1.3 ± 0.4 (0.5–2.0)
Selenium (µg/day)	40.9 ± 27.2 (14.2–96.7)
Vitamin C (mg/day)	56.7 ± 33.4 (22.0–123.0)
Vitamin E (mg/day)	4.9 ± 3.0 (1.2–9.8)
Vitamin A (µg/day)	559.2 ± 275.3 (76.0–928.0)
Tea (ml/day)	717 ± 498 (0–1,425)
Onions (g/day)	4.2 ± 4.5 (0–12.5)

Data are means ± SD (range). %E, percentage of total daily energy intake.

TABLE 2
Flavonol and vitamin content of food supplements (tea and onion dishes) used for the high-flavonol diet

Flavonols and vitamins	Tea (6 mugs; 1,500 ml)		Plain onions (400 g)		Onions, tomato ketchup, and herbs (400 g)	
	µg/ml	mg/day	µg/g	mg/day	µg/g	mg/day
Vitamin A (retinol)	0	0	<0.02	<8 g/day	<0.02	<8 g/day
Vitamin E (α-tocopherol)	0	0	ND	ND	ND	ND
Vitamin C	0	0	3.6	14.4	1.1	4.4
Free quercetin	0.41		5.4		4.20	
Conjugate quercetin	7.04		136.9		221.07	
Total quercetin	7.08	10.0	142.0	57.0	225.37	90.15
Free kaempferol	0.18		0.03		0.03	
Conjugate kaempferol	3.24		0.66		0.98	
Total kaempferol	3.26	4.89	0.72	0.7	1.01	0.41
Free myricetin	ND		ND		ND	
Conjugate myricetin	0.79		ND		ND	
Total myricetin	0.79	0.78	ND	0	ND	0
Free isorhamnetin	ND		0.19		0.17	
Conjugate isorhamnetin	ND		6.03		7.59	
Total isorhamnetin	ND	ND	6.21	2.5	7.78	3.11
Total flavonols	11.14	16.7	148.94	60.2	234.2	93.67

Total daily intake of flavonols provided by the test diet with onion, tomato ketchup, and herbs was 110.37 mg/day, and that with plain onion was 76.3 mg/day. ND, not detectable.

for the whole group. The plasma concentration was numerically higher with the tomato ketchup and onion supplement than with onions alone, but the difference was not significant. Quercetin provided the greatest proportion of flavonols in the supplement (Table 2) and was also the major component of plasma flavonols (Table 3). The supplements of 76.3 or 110.4 mg of flavonols (equivalent to 57 or 90.1 mg quercetin) in addition to a low-flavonol diet therefore increased fasting flavonoid concentrations ~12-fold.

Since the plasma and urine flavonols concentrations were not significantly different between the two high-flavonol diets, subjects were considered as a single high-flavonol group. The scores from the SCGE give a measure of tissue protection against standard oxidative stress. The results showed a significant difference between the low- and high-flavonol diets, supporting the hypothesis that a higher intake and a greater absorption of flavonols are associated with a significantly greater protection against oxidative stress at the tissue level (Fig. 1 and Table 6). The other measures used in this study to assess antioxidant defenses, the endonuclease III assay and the

TEAC assay, showed no difference between the two diets, and both showed relatively high variability (Table 3).

Since many other factors may affect free radical antioxidant systems in the body, strenuous efforts were made to avoid any significant differences between the two diets in their content of other known antioxidant systems. The data in Tables 4 and 5 show no change between high- and low-flavonol diets in any of the antioxidant vitamins or carotenoids, or in selenium, superoxide dismutase, or glutathione peroxidase. There were no changes in plasma urate, albumin, or bilirubin, all known to be powerful endogenous antioxidants. Plasma fructosamine was 320 µmol/l on the low-flavonol diet and 323 µmol/l with supplements, so the better antioxidant activity cannot be attributed to any improvement in diabetic control.

DISCUSSION

Flavonols have been considered for >60 years to be potentially beneficial components of fruits and vegetables. Their importance first came to light when a vitamin C sparing effect was

TABLE 3
Plasma and urine flavonol responses of type 2 diabetic patients to a high-flavonol diet based on the SCGE assay to measure protection of fresh lymphocytes against H₂O₂ damage to DNA and the endonuclease III assay for endogenous DNA damage analysis

	Low-flavonol diet	High-flavonol diet	P value
Fasting plasma quercetin (ng/ml)	5.6 ± 2.9	67.8 ± 15.2	<0.005
Fasting plasma total flavonols (ng/ml)	5.6 ± 2.9	72.1 ± 15.7	<0.005
24-h urine quercetin concentration (ng/ml)	12.5 ± 5.2	112.4 ± 17.7	<0.0005
24-h urine total flavonols concentration (ng/ml)	15.2 ± 6.2	148.9 ± 20.7	0.0001
24-h urine quercetin excretions (g/day)	17.3 ± 7.5	218 ± 52.5	<0.005
24-h urine total flavonol excretion (g/day)	21.2 ± 9.0	281.8 ± 59.1	<0.005
SCGE (out of 400)	220 ± 12.0	191.5 ± 13.5	0.037
Endonuclease III (out of 400)	82.4 ± 4.5	91.4 ± 10.9	0.42
TEAC(mmol/l)	1.40 ± 0.03	1.44 ± 0.03	0.22

Data are means ± SE (n = 10).

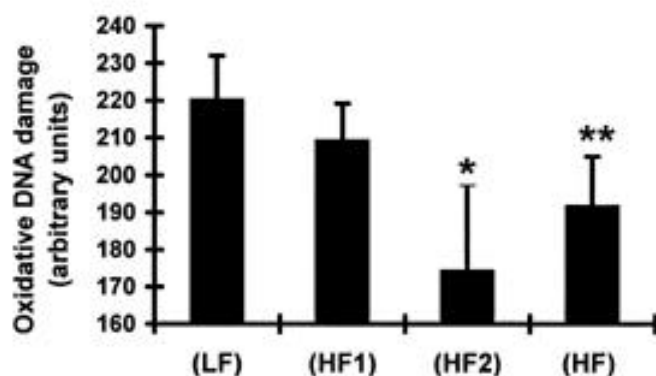


FIG. 1. The effect of low-flavonol (LF) and high-flavonol (HF) diets. HF (HF1 and HF2 combined), for protection against oxidative DNA damage in lymphocytes of type 2 diabetic patients assessed by the Comet assay. HF1, onion, tomato ketchup, and herb; HF2, plain onion. Results are expressed as means \pm SE for $n = 10$. * $P < 0.23$ (HF2 vs. HF1); ** $P < 0.05$ (LF vs. HF) and $n = 5$.

observed (29). In vitro work has suggested a number of potentially important functions for flavonols. Their antioxidant activity is of particular importance, notably in the protection against LDL oxidation, a key process in the pathogenesis of atherosclerosis (30). Data from the Netherlands suggest that flavonols are present in the diet at levels on the order of 23 mg/day, mostly in the form of quercetin, and largely obtained from tea (61%), onions (13%), and apples (10%) (17). Much larger daily intakes might be expected in high consumers of these foods, and there are consistent differences between the flavonol contents of distinct varieties of fruits and vegetables (25).

Until recently, there was very little information available on whether dietary flavonoids, particularly flavonols, are absorbable. Early data suggested that the conjugated flavonols, in contrast to aglycone, were precluded from intestinal absorption (31). However, acute dosing experiments have recently indicated the opposite, i.e., greater absorption of conjugated flavonols and minimal absorption of aglycone (19,32,33), with an elevation of plasma flavonols for 1–5 h after dosing. The present study is the first to examine the extended treatment of high-flavonol supplements and relate this to measurements of protection against oxidative stress at a tissue level. Very clear evidence has emerged showing significant absorption of dietary flavonols, specifically quercetin and its conjugates, with a mean increase in

fasting plasma flavonol concentrations of ~12-fold from a relatively small supplement.

Several studies have demonstrated improved antioxidant defenses in subjects given foods or diets that might contain increased flavonoids, but usually without evidence that flavonoids are absorbed and are thus responsible for the improvement observed. The potential confounding effects from other antioxidant factors may not have been rigorously excluded in all instances. The acute consumption study of McAnlis (33) showed no effect from 225 g of onions on the resistance of plasma to copper-induced oxidation. This test is similar to the plasma TEAC assay used in the present study, which also showed no significant difference in the fasting antioxidant capacity of plasma between high- and low-flavonol diets over 28 days. These tests are relatively crude, are affected by many endogenous factors, and may not relate directly to free radical-mediated damage within cells: 50 μ m quercetin was necessary to increase the TEAC of plasma in vitro (M.N., H. Millar, N.S., M.E.J.L., unpublished observations). Our use of SCGE on fresh lymphocytes to assess the result of a dietary intervention was a novel approach and showed, at a tissue level, a significant increase in the protection against DNA damage from H_2O_2 . We have previously used the SCGE assay to study the antioxidant effect of preincubation with flavonoids, including flavonols, and have found dose-dependent effects with all common flavonoids, most significantly more potent than vitamin C (M.N., H. Millar, N.S., M.E.J.L., unpublished observations; 20). The endonuclease III method did not show any change in endogenous DNA damage, but the present study was probably too short, at 2 weeks, and subjects did not experience acute oxidative stress.

Differences between diabetic and nondiabetic subjects were not sought in the present study, but the Comet assay might be used for this purpose in future work. There is growing awareness that free radical processes may be of particular importance in the microvascular and macrovascular complications of diabetes (34). Abnormal antioxidant status is found in the prediabetic state of impaired glucose tolerance (IGT), and this may contribute to the high coronary heart disease risk in IGT patients (35). Decreased lipid peroxidation and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications (36). Excess DNA damage, indicated by higher 8-hydroxy-2'-deoxyguanosine excretion than in nondiabetic control subjects, has been reported in both type 1 and

TABLE 4
Plasma and urine measurements of antioxidant factors on high- and low-flavonol diets

	Low-flavonol diet	High-flavonol diet	P value
Superoxide dismutase activity (U/ml)	0.03 \pm 0.01	0.05 \pm 0.02	0.30
Gluthathione peroxidase (U/l)	210.3 \pm 12.8	213.0 \pm 8.8	0.86
Selenium (μ mol/l)	1.28 \pm 0.10	1.20 \pm 0.08	0.36
Plasma albumin (g/l)	45.1 \pm 0.8	45.9 \pm 0.6	0.69
Urine albumin (mg/l)	13.4 \pm 4.6	16.4 \pm 6.4	0.12
Plasma globulin (g/l)	30.8 \pm 1.2	30.0 \pm 1.0	0.74
Total plasma protein (g/l)	72.8 \pm 3.7	76.0 \pm 1.7	0.86
Plasma bilirubin (U/l)	13.8 \pm 2.8	15.6 \pm 3.3	0.83
Fructosamine (μ mol/l)	320 \pm 22	323 \pm 20	0.72

Data are means \pm SE ($n = 10$).

TABLE 5
Plasma antioxidant vitamin and carotenoids on low- and high-flavonoid diets

	Low-flavonoid diet	High-flavonoid diet	P value
Vitamin A (µmol/l)	2.6 ± 0.5	2.7 ± 0.4	0.28
Vitamin C (mmol/l)	35.7 ± 3.4	30.1 ± 3.2	0.13
Vitamin E (µmol/l)	42.0 ± 5.5	45.7 ± 6.1	0.11
Vitamin E/cholesterol*	6.7 ± 0.7	7.2 ± 0.6	0.15
β-Carotene (µmol/l)	0.16 ± 0.03	0.16 ± 0.04	0.95
β-Carotene/cholesterol*	0.028 ± 0.007	0.027 ± 0.008	0.47
β-Cryptoxanthine (µmol/l)	0.04 ± 0.01	0.04 ± 0.01	0.39
β-Cryptoxanthine/cholesterol*	0.006 ± 0.001	0.006 ± 0.001	1.00
Lycopene (µmol/l)	0.197 ± 0.039	0.244 ± 0.059	0.22
Lycopene/cholesterol*	0.034 ± 0.007	0.042 ± 0.011	0.22
Total carotenoids (µmol/l)	0.605 ± 0.067	0.652 ± 0.103	0.44
Total carotenoids/cholesterol*	0.104 ± 0.016	0.112 ± 0.020	0.38
Lutein (µmol/l)	0.16 ± 0.02	0.15 ± 0.02	0.57
Lutein/cholesterol*	0.03 ± 0.004	0.03 ± 0.005	1.00
α-Carotene (µmol/l)	0.05 ± 0.008	0.05 ± 0.01	0.62
α-Carotene/cholesterol*	0.009 ± 0.002	0.009 ± 0.002	0.17
Cholesterol (mmol/l)	6.3 ± 0.6	6.3 ± 0.6	1.00

Data are means ± SE (n = 10). *Ratio of species to plasma cholesterol (×10³ = mmol/l). Laboratory reference ranges: vitamin A 1.4–2.6 µmol/l; vitamin E 22–37.2 µmol/l; vitamin C 11–114 µmol/l; lutein 0.15–0.37 µmol/l; lycopene 0.19–0.55 µmol/l; α-carotene 0.03–0.11 µmol/l; β-carotene 0.18–0.58 µmol/l; β-cryptoxanthine 0.14–0.36 µmol/l.

type 2 diabetic patients (37,38). Sinclair et al. (11) have reported that there is a negative correlation between serum ascorbic acid and fructosamine concentration in diabetic patients with complications. This group has also reported a low concentration of plasma ascorbate in patients with type 2 diabetes consuming adequate dietary vitamin C, and suggested that this implies increased use of vitamin C to inactivate free radicals.

Dietary recommendations for diabetes encourage high fruit and vegetable intakes (39). The present study provides further evidence to justify this recommendation, primarily aimed at reducing cardiovascular disease. Very few studies in diabetic subjects have sought improvements from administration of known antioxidants, although claims have been made for high doses of vitamins C and E (40). In vitro

SCGE studies, we have shown that the effect of flavonols is additive to that of vitamin C, and on this basis, it would seem appropriate to suggest that diets relatively high in flavonols as well as conventional antioxidant vitamins should be recommended for patients with diabetes. It might be hypothesized that diabetic patients with a high intake of flavonol-rich foods, and specifically onions, might be relatively protected against long-term complications. As far as we are aware, evidence for such an effect does not exist at present, but appropriate analyses of large databases might be encouraged to seek supporting evidence of this kind.

Future work should address the impact of dietary flavonoids, including flavonols, on other indices of free radical-mediated disease and should identify dietary measures that will increase consumption of absorbable flavonols in the long term.

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TABLE 6
Endogenous protection of fresh lymphocytes against oxidative DNA damage from hydrogen peroxide after low- and high-flavonol diets, estimated by the Comet assay

Subject	Low-flavonol diet	High-flavonol diet
1	164	201
2	236	208
3	211	172
4	259	205
5	223	217
6	205	100
7	264	220
8	253	232
9	153	137
10	236	224
Mean ± SE	220 ± 12	191.5 ± 13.5

Data represent oxidative DNA damage in arbitrary units, out of 400. Each unit is the mean of three triplicate measurements.

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Author Queries (please see Q in margin and underlined text)

Q1: Changes to sentence beginning “The dietary supplement...” OK? If not, please reword for clarity.

Q2: Changes to sentence beginning “Patients were advised...” OK? If not, please reword for clarity.

Q3: Please provide location for Gibco.

Q4: Randix: Please provide manufacturer name and location.

Q5: RF-10AXL fluorimeter: Please provide manufacturer name and location.

Q6: Please provide location for Roche.

Q7: Table 4 has been split into two tables, Table 4 and Table 5. Please correct Table numbers in call-outs. Tables must be called out in numerical order. Would you like to include a call-out for Tables 4 and 5 before this one for Table 6 or to renumber the tables?

Q8: Au: Changes to figure legend OK? If not, please reword for clarity.

Q9: McAnlis (and not McCanlis) correct here, as in references?

Refs. 3, 29, 39: Is this a letter, an abstract, or a one-page article? If not, please provide full page range.

Au: Refs. 20, 26, 33: Please provide updated publication information.

Refs. 16 and 32 have been made into in-text citations per journal style. If these articles have been published, please provide updated information.

Table 2: Correct that ND stands for “not determined”?