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Reduction in Free-Radical-Induced DNA Strand Breaks and Base Damage through Fast Chemical Repair by Flavonoids

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This paper provides evidence that dietary flavonoids can repair a range of oxidative radical damages on DNA, and thus give protection against radical-induced strand breaks and base alterations. We have irradiated dilute aqueous solutions of plasmid DNA in the absence and presence of flavonoids (F) in a "constant $^{\bullet}$OH radical scavenging environment", k of 1.5×10^7 s^{-1} by decreasing the concentration of TRIS buffer in relation to the concentration of added flavonoids. We have shown that the flavonoids can reduce the incidence of single-strand breaks in double-stranded DNA as well as residual base damage (assayed as additional single-strand breaks upon post-irradiation incubation with endonucleases) with dose modification factors of up to 2.0 ± 0.2 at [F] $< 100\,\mu$M by a mechanism other than through direct scavenging of $^{\bullet}$OH radicals. Pulse radiolysis measurements support the mechanism of electron transfer or H$^{\bullet}$ atom transfer from the flavonoids to free radical sites on DNA which result in the fast chemical repair of some of the oxidative damage on DNA resulting from $^{\bullet}$OH radical attack. These *in vitro* assays point to a possible additional role for antioxidants in reducing DNA damage.

Keywords: Flavonoids, antioxidants, electron transfer, DNA strand breaks, base damage, pulse radiolysis

Abbreviations: CHD, coronary heart disease; LDL, low density lipoproteins; TRIS, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DMF, dose modification factor; FPG, formidopyrimidine-DNA glycosylase; EndoIII, endonuclease III; ExoIII, exonuclease III; DMSO, dimethylsulfoxide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; F, flavonoid; *E*(1), one-electron reduction potential; "TEAC", Trolox equivalent antioxidant potential; Trolox®, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

INTRODUCTION

Dietary flavonoids are presently under scrutiny for their possible health benefits. Epidemiological studies indicate an inverse relationship between the intake of flavonoids and coronary heart disease, CHD,[1–3] and a high intake of fruits and vegetables, the source of dietary flavonoids, is also thought to reduce the incidence of cancer.[4–6] Although the average Western diet may provide

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up to 1 g of flavonoids per day[7] only small concentrations of the flavonoids, from micromolar to tens of micromolar (including derivatives such as the glucuronides) can be detected in human plasma even after supplementation.[8,9] Flavonoid concentrations used in *in vitro* studies which result in the inhibition of LDL oxidation and platelet aggregation[10,11] are 10–1000 times higher than the levels reached in human plasma. This disparity has lead to the significance of the flavonoids in effecting the amelioration of CHD by these mechanisms to be questioned.[12] Flavonoids have been shown to induce single DNA strand breaks in rat liver nuclei[13] and in cultured human cells, but at high concentrations[14] which are unachievable in humans.

Flavonoids are efficient scavengers of free radicals *per se*[15–17] and are effective in scavenging lipid peroxy radicals thereby inhibiting peroxidation.[18,19] Flavonoids also inhibit the peroxynitrite-mediated formation of 8-oxodeoxyguanosine,[20] a marker for oxidative damage sustained by DNA. A study on the protection of plasmid pBR322 DNA by flavonoids against single-strand breaks (ssb) induced by singlet molecular oxygen was ascribed to competitive quenching.[21] However, these effects have also been demonstrated in *in vitro* systems using high concentrations of the flavonoids. The protective effect of several flavonoids in inhibiting oxygen radical-generated DNA damage to human lymphocytes using the comet assay has been reported to be greater than that of vitamin C[22] and a reduction in the rate of oxidative DNA-damage in humans has been found following the ingestion of Brussels sprouts[23] which contain phytochemicals including high levels of flavonoids. As oxidative DNA damage is considered to be a pathogenic event in the induction of many cancers,[24,25] a reduction in the rate of such damage may indicate a reduced risk of cancer. We are studying possible mechanisms of how low concentrations of the flavonoids could lead to health benefits in humans and report evidence for fast chemical repair of oxidative damage to DNA through electron transfer based on the results from pulse radiolysis studies and a plasmid test system. Our studies are centred on the reaction of $^{\bullet}OH$ radicals with DNA as the $^{\bullet}OH$ radical is the major oxidising species which induces strand breaks[26,27] and causes modifications in DNA including base lesions.[28,29]

Pulse radiolysis studies have provided both spectral[30–32] and redox[33,34] information on DNA base radicals formed upon one-electron oxidation. Fast chemical repair by antioxidants of the guanyl radical formed upon the reaction of the $^{\bullet}OH$ radicals with guanosine and dGMP[30,35–38] has been demonstrated. Most attention has been paid to the repair of the guanyl radical as guanine is the most easily oxidised of the DNA bases. While electron transfer from certain antioxidants to radical sites on single-stranded DNA has been reported,[37] the efficacy of such an antioxidant reaction in reducing the amount of strand breakage in double-stranded DNA, both in the presence and absence of oxygen, has not been studied. Dietary flavonoids, such as catechin, epicatechin, quercetin, fiestin, kaempferol, luteolin and apigenin, are attractive candidates for such an antioxidant reaction as the one-electron reduction potentials of their radical cations[39,40] are lower than the radical sites on DNA. Conversion of supercoiled double-stranded DNA (Form I) to its relaxed coiled form (Form II) and the separation of the forms by gel electrophoresis[41] is a convenient and widely used method to study DNA damage. Direct (or prompt) DNA strand breakage arising from free radicals produced upon the γ-irradiation of water can be assayed as well as a range in base damages which are recognised upon treatment with certain endonucleases[42–44] to form additional strand breaks.

MATERIALS AND METHODS

All chemicals were of the highest analytical grade available and used as received. The flavonoids, Figure 1, were obtained from various suppliers;

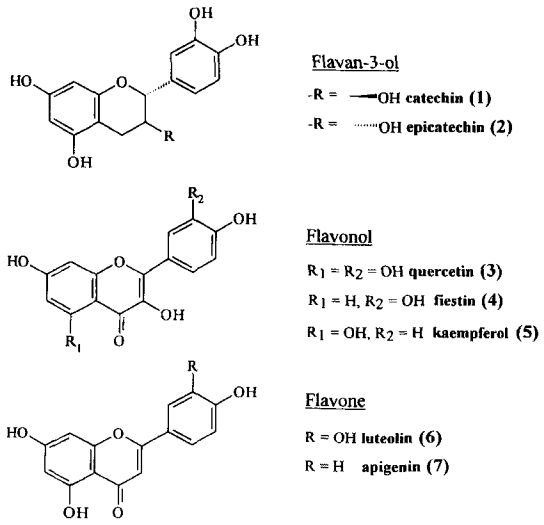

FIGURE 1 Structures of the flavonoids used in this study.

catechin trihydrate, (−)-epicatechin, quercetin dihydrate, kaempferol (ICN Biomedicals Inc.), luteolin, apigenin (Lancaster Synthesis) and fiestin (ACROS).

Stock solutions of calf thymus DNA (Sigma-Aldrich), used as carrier DNA and in the pulse radiolysis experiments, were prepared by firstly dissolving the DNA in stirred "Milli-Q" water followed by shearing 3× through a 23G needle, sonication, and finally dialysis against a storage buffer solution of 0.1 mM EDTA and 1 mM phosphate, pH 7.4. Plasmid DNA, pBR322 was prepared by expression in *Escherichia coli*, followed by extraction and purification using a Qiagen Plasmid Maxi-kit (Qiagen GmbH, Germany). All preparations contained >92% of the intact closed circular form (Form I), the balance being the relaxed coiled form (Form II). Plasmid DNA Forms I and II were separated on agarose gels (1%) by electrophoresis in TAE buffer. Quantification of the bands following ethidium bromide staining (applying a calibrated enhancement of 1.3 to the closed circular image) was done using the NIH image software package. Southern blotting was performed in experiments when calf thymus DNA was also present at the time of irradiation, followed by the non-radioactive DIG detection to identify the plasmid bands. Test solutions of plasmid ($12\,\mu g\,mL^{-1} = 4.23\,nM$ using relative molecular mass $= 650\,g\,mol^{-1}\,bp^{-1} \times$ number of base pairs) and added flavonoids were irradiated in the presence of air using a 1.5×10^4 GBq ^{60}Co γ-ray source providing a dose rate of $42\,Gy\,min^{-1}$ (Fricke dosimetry) at room temperature (22°C). Irradiated samples were withdrawn serially and kept on ice until subsequent analysis. Under these conditions the radiation breaks down the water into quantified amounts (in $\mu mol\,J^{-1}$) of free radicals of which the $^{\bullet}OH$ radical is the main oxidising species with the reducing H atoms and e^-_{aq} being converted to superoxide.

$$H_2O \rightsquigarrow {}^{\bullet}OH(0.28),\ H^{\bullet}(0.06),\ e^-_{aq}(0.28)\ (+H_3O^+,\ H_2O_2,\ H_2) \quad (1)$$

$$e^-_{aq}/H^{\bullet} + O_2 \rightarrow O_2^{\bullet -}/(HO_2^{\bullet}) \quad (2)$$

DNA ssb induced in the plasmid (conversion of Form I to II) following irradiation in the presence and absence of added flavonoids, were determined using the plasmid dissolved in TRIS buffer (≤ 10 mM, pH 7.4) containing phosphate (1 mM) and EDTA (0.1 mM). The influence of added flavonoids, F, on free radical damage to the plasmid was studied at a constant scavenging capacity, k, which we define as the summation of the pseudo first-order rate constants, k_x for the reaction of $^{\bullet}OH$ radicals with each component, x, in solution multiplied by its concentrations; i.e. $k = \sum_1^n k_x[x]_n$ (in s^{-1}). Control experiments were carried out in 10 mM TRIS buffer, where $k_3 = 1.5 \times 10^9\,M^{-1}\,s^{-1}$ [45] and hence $k = 1.5 \times 10^7\,s^{-1}$.

$$^{\bullet}OH + TRIS \rightarrow TRIS^{\bullet} + H_2O \quad (3)$$

$$^{\bullet}OH + F \rightarrow F^{+\bullet} + OH^- \quad (4)$$

The scavenging capacity was maintained in the presence of added flavonoids, by adjusting the concentration of the TRIS buffer according to [TRIS] (in M) $= (1.5 \times 10^7\,s^{-1} - [F] \times k_4)/k_3$ (the very small test concentrations of plasmid used

as well as the phosphate buffer and EDTA do not contribute significantly to k compared to TRIS and the flavonoids). By maintaining a constant k, we reason that the proportionate protection of the plasmid from ssb formation and other damage through direct radical scavenging (i.e. intercepting $^{\bullet}OH$ radicals by TRIS buffer and the flavonoids before they react with DNA) is similar in the controls as for when flavonoids are also present. Hence the possible subsequent effect of the flavonoids on the DNA radicals in terms of altering the yield of DNA damage can be studied.

The D_0 values for the loss of Form I due to ssb formation were obtained from the slopes of the regression lines of the percentage of Form I as a function of dose using $D_0 = ((\log_{10} 37) - 2)/\text{slope}$ where D_0 is the radiation dose required on average to produce one strand break in Form I. The ratios of the D_0 values to that of the control (10 mM TRIS buffer) were used to calculate dose modification factors (DMF) for each flavonoid concentration and also determining the G value (radiation chemical yield) for ssb formation in $\mu mol\ J^{-1}$ where $G(ssb) = [DNA]\ (in\ \mu M)/D_0$ (in Gy) $\times\ \rho$ ($kg\ L^{-1}$, assumed to be unity).

DNA base damage was evaluated as increased amount of strand breakage (ess) formed upon incubating irradiated plasmid (in the presence and absence of flavonoid) with the DNA glycosylases formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (endoIII) which recognise a wide range of damage to purine bases, pyrimidine bases, and also with the 5′ AP endonuclease exonuclease III (exoIII) which recognises AP (apurine/apyrimidine) sites.[46,47] EndoIII protein was kindly supplied by Dr. R.P. Cunningham (SUNY), Fpg protein was purified from an overexpressing strain of *E. coli* kindly gifted by Dr. Y.W. Kow (Emory University School of Medicine) and exoIII was purchased from Boerhinger Mannheim, Germany. Irradiated samples were incubated with each protein, prior to gel electrophoresis, in a total volume of 20 μL for 30 min at 37°C with either (i) Fpg (30 ng) in 20 mM TRIS–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, (ii) endoIII (3 ng) in 20 mM TRIS–HCl, pH 7.5, 100 mM NaCl, 15 mM EDTA, or (iii) exoIII (5U) in 20 mM TRIS–HCl, pH 7.5, 100 mM NaCl, 15 mM $CaCl_2$, as well as in the buffers alone. The protein concentrations used in the incubation experiments were at the lowest concentrations found to be capable of producing maxima in the cutting of damaged DNA. This was done to avoid observed nuclease activities at high concentrations of the protein preparations on unirradiated control samples.

Pulse radiolysis and its associated dosimetry were carried out using the University of Auckland's facility.[48] Pulses of electrons (typically 3 Gy ($J\ kg^{-1}$) absorbed dose in 0.2 μs) were used to initiate radical reactions in a 1 cm quartz cell. Time-resolved spectra were normalised for radiation dose and the $^{\bullet}OH$ radical yields calculated using a total radiation chemical yield, G, of $0.6\ \mu mol\ J^{-1}$ in N_2O (as well as N_2O/O_2, 4/1) saturated solutions, and a G of $0.29\ \mu mol\ J^{-1}$ in both N_2-saturated and aerobic solutions. Use of N_2O quantitatively converts the e^-_{aq} into $^{\bullet}OH$ radicals,

$$e^-_{aq} + N_2O \rightarrow {}^{\bullet}OH + OH^- + N_2 \qquad (5)$$

Pulse radiolysis was used to determine thermodynamically reversible $E(1)$ values relative to a reference couple ($R^{+\bullet}/R$).[39]

$$A^{+\bullet} + R \leftrightarrows A + R^{+\bullet}$$

where $K = [A]/[R]\{(OD_{eq} - OD^{+\bullet}_R)/(OD^{+\bullet}_A - OD_{eq})\}$ and $OD^{+\bullet}_A$, $OD^{+\bullet}_R$, OD_{eq} are the observed absorbances normalised for radiation dose for the antioxidant, reference compound and at equilibrium, respectively. As $\Delta E = -(RT/F)\ln K$, $E(1)\ A^{+\bullet}/A$ can be determined.

RESULTS

The rate constants for $^{\bullet}OH$ radical with the flavonoids, k_4, at pH 7.4 (1 mM phospate buffer) were

determined from competition plots derived from the decrease in the flavonoid radical absorption, $F^{+\bullet}$, produced in the presence of increasing concentrations of dimethylsulphoxide (DMSO) or the formation in the absorption of the radical cation of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) at 560 nm assuming a second-order rate constant with $^{\bullet}OH$ radicals of $k_6 = 6.6 \times 10^9\,M^{-1}\,s^{-1}$ for the reaction of DMSO with $^{\bullet}OH$ radical[45] and $k_7 = 1.0 \times 10^{10}\,M^{-1}\,s^{-1}$ with TMPD (determined against the oxidation of ferrocyanide, where k ($^{\bullet}OH$ + ferrocyanide) $= 1.05 \times 10^{10}\,M^{-1}\,s^{-1}$.[45]

$$^{\bullet}OH + (CH_3)_2SO \rightarrow (CH_3)_2S^{\bullet}O(OH) \quad (6)$$

$$^{\bullet}OH + TMPD \rightarrow TMPD^{+\bullet} + H_2O \quad (7)$$

The second-order rate constants for the reaction of $^{\bullet}OH$ radicals with the flavonoids (Table I) were determined from competition kinetic plots for at least three mixtures of flavonoid (0.1–0.2 mM) and DMSO or TMPD (0.2–0.6 mM) and the values used in preparing solutions of the plasmid for irradiation. The determined rate constants, at pH 7.4, are somewhat greater than those reported for measurement made at pH 11.5.[49]

DNA strand breaks in the presence and absence of the flavonoids under various conditions were assessed following irradiation of the plasmid in mainly aerobic solutions with a small study in hypoxia for comparison purposes. The radicals produced on the breakdown of water undergo radical–radical reactions and reactions with added substrates in dilute solution. By maintaining a low constant radical scavenging environment, strand breakage arising from the direct ionisation of the DNA is a very minor component of the observed dose related strand breaks.[26,50] The observed strand breaks almost exclusively arise directly or indirectly from reactions initiated by a small proportion of the $^{\bullet}OH$ radical yield, produced on the irradiation of water, which is formed in close proximity to the DNA,[50]

$$^{\bullet}OH + DNA + H^+ \rightarrow H_2O + DNA^{+\bullet} \rightarrow\rightarrow \text{strand break} \quad (8)$$

The radiolytic conversion of Form I to II, both with and without added flavonoids, was followed using gel electrophoresis. Typical dose response curves for the loss Form I under both aerobic and hypoxic conditions as a function of catechin

TABLE I Physicochemical properties of flavonoids

Compound	$E(1)$ ($F^{+\bullet}/F$) (in V)	$10^{-10}k_2$($^{\bullet}OH$ + F) (in L mol^{-1} s^{-1})	DMF_{max}	$10^{-3}k_9$ (in s^{-1}), fast[e] (% ET_{N_2O})[f]	$10^{-3}k_9$ (in s^{-1}), fast[e] (% ET_{N_2O/O_2})[f]	k_{11} (in s^{-1}), slow[e] (% ET_{N_2O/O_2})[f,g]
Catechin (**1**)	0.57 ± 0.02[a,d]	1.5 ± 0.2	2.0 ± 0.2	3.5 ± 0.2 (25 ± 2)	2.9 ± 0.5 (12 ± 2)	155 ± 5 (31 ± 2)
Catechin (**1**) + $NaClO_4$		1.5 ± 0.2	2.0 ± 0.2	5.0 ± 0.2 (29 ± 2)	3.0 ± 0.2 (13 ± 1)	195 ± 5 (29 ± 2)
Epicatechin (**2**)	(0.57)[b]	1.5 ± 0.1	2.1 ± 0.2	2.6 ± 0.3 (18 ± 2)	2.2 ± 0.2 (10 ± 1)	150 ± 22 (26 ± 2)
Quercetin (**3**)	0.60 ± 0.02[a]	1.0 ± 0.2	1.7 ± 0.1	2.0 ± 0.2 (10 ± 2)	1.1 ± 0.2 (10 ± 2)	160 ± 10 (29 ± 2)
Fiestin (**4**)	[c]	1.1 ± 0.2	1.7 ± 0.2			
Luteolin (**6**)	0.72 ± 0.01[d]	0.9 ± 0.2	2.2 ± 0.2	2.3 ± 0.2 (5 ± 1)	2.0 ± 0.2 (4 ± 1)	170 ± 10 (17 ± 2)
Kaempferol (**5**)	0.95[a]	1.2 ± 0.1	1.4 ± 0.1	2.2 ± 0.2 (5 ± 1)	1.0 ± 0 (5 ± 1)	100 ± 12 (11 ± 1)
Apigenin (**7**)	1.04 ± 0.01[d]	1.2 ± 0.1	1.4 ± 0.2	< 0.1 (0)	< 0.1 (0)	90 ± 10 (7 ± 1)

[a]Ref. [40], [b]taken as that for catechin, [c]not determined, [d]this work, [e]value at [DNA] = 2.0 mM and [F] = 40 μM, [f]maximum level of flavonoid radical formed, [g]total yield of fast and slow phases in N_2O/O_2.

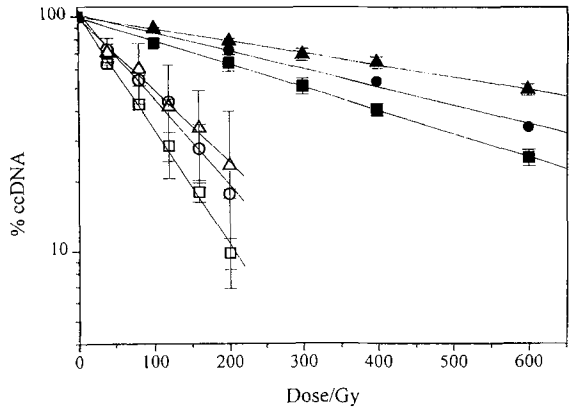

FIGURE 2 Effect of catechin on the radiation dose-dependent loss of closed-circular fraction of plasmid DNA ($12\,\mu g\,mL^{-1}$) upon the γ-irradiation of anaerobic (open symbols) and aerobic (closed symbols) solutions containing: (i) TRIS buffer (10 mM), □, ■; (ii) catechin (10 μM) + TRIS buffer (9.9 mM), ○, ●; and (iii) catechin (100 μM) + TRIS buffer (9.0 mM), △, ▲. Data points are the average of three separate experiments with error bars representing the standard deviation. Solid lines are fitted by regression analysis.

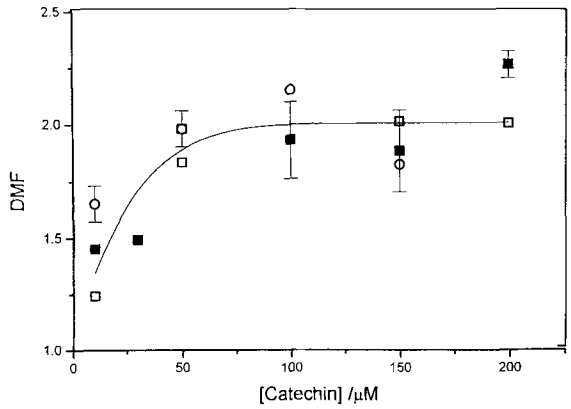

FIGURE 3 Dose modification factors (DMF) for the protection of closed-circular plasmid DNA by increasing concentrations of catechin upon the γ-irradiation of aerobic solutions at a constant scavenging capacity, k of $1.5 \times 10^7\,s^{-1}$. Solutions contained (i) TRIS buffer, ■; (ii) TRIS buffer + sodium perchorate (0.15 M) □; and (iii) TRIS buffer + calf thymus DNA (0.5 mM base pairs), ○.

concentration at a constant k of $1.5 \times 10^7\,s^{-1}$ are presented in Figure 2. The D_0 values derived from the slopes of the curves in Figure 2 are used to calculate DMF values at each catechin concentration. Catechin is observed to give some protection against DNA strand breaks in a dose dependent manner under aerobic and hypoxia conditions, where it is known that *in vitro* DNA is more sensitive to irradiation.[50] Figure 3 summarises the DMF values determined for increasing concentrations of catechin tested in aerobic solutions containing (i) TRIS buffer, (ii) TRIS buffer and sodium perchorate (0.15 M) and (iii) TRIS buffer and calf thymus carrier DNA (0.5 mM base pairs). In all cases the D_0 values of the controls did not change significantly from that under conditions of TRIS buffer alone. Under all conditions the DMF values are > 1.0 even at the lowest concentration of catechin tested (10 μM) rising to a plateau value of *ca.* 2.0 which corresponds to a halving of the G value for ssb. Dose response curves are restricted to ≤ 600 Gy at which level $< 20\%$ of the added concentrations of catechin are calculated to have been consumed by $^{\bullet}OH$ radicals produced by the radiation. Addition of the calf thymus DNA effectively provides a large number of possible DNA binding sites (several orders in magnitude) over the test plasmid DNA and the high concentration of sodium perchlorate salt would disrupt any ionic binding of the flavonoids to DNA. The fact that DMF values are not reduced in the presence of carrier DNA or when salt is added, suggests that catechin is not exerting its effect by either binding or associating strongly with DNA. A plateau in DMF value is observed in Figure 3, which implies that there is a saturable amount of DNA lesions which can be affected by catechin. All other flavonoids tested gave similar results, except apigenin and kaempferol which exhibited lower plateau DMF values (Table I).

The effect of catechin (100 μM) in possibly reducing free-radical-induced base damage in addition to reducing DNA strand breaks was studied by post-irradiation incubation of serially dosed samples of the plasmid with endoII, Fpg and exoIII proteins which recognise a range of DNA base damage by cutting the DNA. Control samples were subjected to the same post-irradiation incubation conditions (heating, incubation time and buffers) with and without added

catechin in the absence of the proteins. Incubation of irradiated plasmid with all three proteins induced increases in the level of strand breakage, ess, over non-enzyme treated controls, observed as increased loss of Form I with radiation dose, Figure 4. The partial protection from strand breakage afforded by catechin was maintained when irradiated samples were subjected to post-irradiation incubation with the endonucleases but to different extents for each protein. The fact that all of the proteins cut the irradiated DNA to a large extent despite the presence of a high concentration of catechin at the time of irradiation indicates that the flavonoid is not acting to completely prevent residual damage to the pyrimidine and purine bases or in the formation of AP sites. The effectiveness of catechin in ameliorating oxidative damage which is recognised by each protein can be ascertained from Figure 4 by comparing the *G* values for ess (endonuclease-sensitive sites leading to increased strand breakage due to the action of the enzymes over direct strand breakage, ssb) in the absence and presence of the flavonoid. The difference in *G* values between the control and protein-treated cases gives the *G* (ess) value for the increase in strand breakage arising from the action of each protein while this difference when catechin is present at the time of irradiation gives the antioxidant-induced reduction in strand breaks and base damage which is recognised by the proteins.

Subtracting this second calculated value from *G* (ess) yields the *G* value for base damage (recognised by the proteins) which is reduced by the catechin, Figure 5. The *G* values for AP sites recognised by the exoIII protein is less than the *G* value for direct strand breaks whereas the *G* values for recognisable purine and pyrimidine base damage (FPG and endoIII proteins) are in excess of that for direct strand breaks.

Pulse radiolysis experiments were carried out to gain some insight into the reactions between

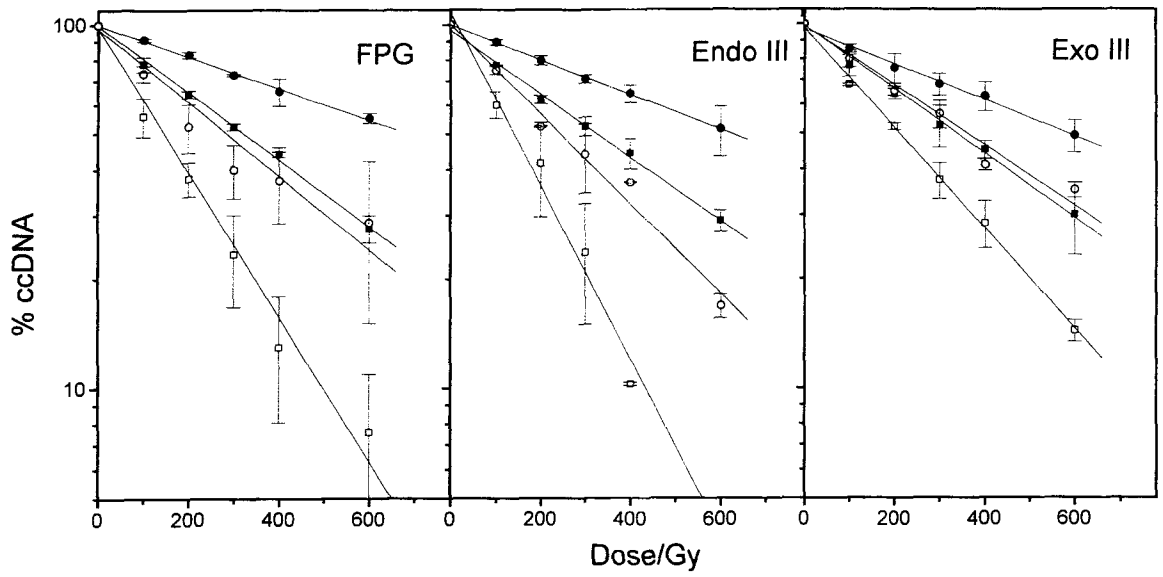

FIGURE 4 Effect of catechin (100 μM) on the radiation dose-dependent loss of closed-circular fraction of plasmid DNA (12 μg mL^{-1}) upon the γ-irradiation of aerobic solutions and subsequent incubation with and without the endonucleases: (i) FPG (formidopyrimidine-DNA glycosylase); (ii) EndoIII (endonuclease III); and (iii) ExoIII (exonuclease III). TRIS buffer control, ■; TRIS buffer + catechin, ●; TRIS buffer + post-irradiation incubation with endonucleases, □; TRIS buffer + catechin + post-irradiation incubation with endonucleases, ○. Data points are the average of three separate experiments with error bars representing the standard deviation. Solid lines are fitted by regression analysis.

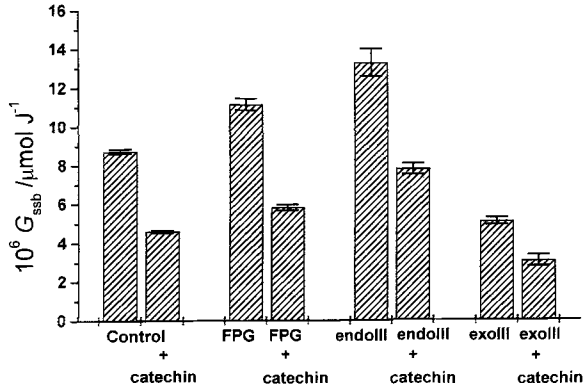

FIGURE 5 Yields of DNA strand breaks and their reduction in the presence of catechin (100 µM) due to the direct action of the •OH radicals, and the formation of additional DNA strand breaks upon incubation with FPG, endoIII and exoIII nucleases which recognise a variety of residual base damages. Error bars represent the standard deviation derived from the average of the D_0 values.

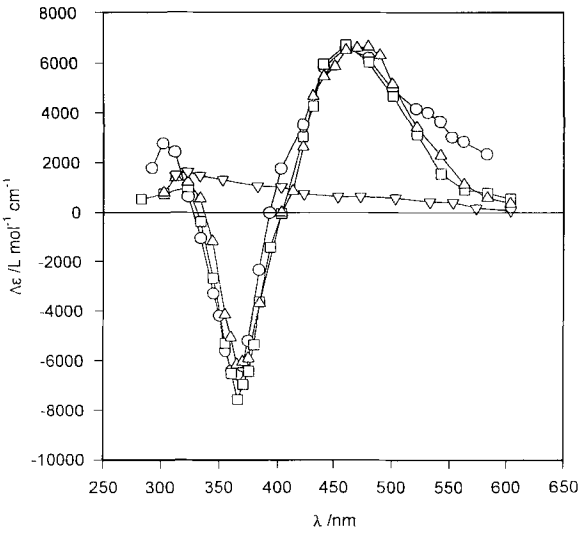

FIGURE 6 Transient absorption spectra (radicals minus the absorption of unirradiated parent compound) in N_2O-saturated phosphate buffer (5 mM, pH 7.4) following pulse radiolysis (3 Gy in 200 ns) of: (i) DNA (2 mM, base pairs), ▽; (ii) luteolin (100 µM), ○; (iii) luteolin (100 µM) + selenite (50 mM), □; and (iv) luteolin (100 µM) + selenite (50 mM) + deoxyguanosine (1 mM), △. Changes in extinction coefficient calculated assuming a radiation chemical yield, G, of 0.6 µmol J^{-1}.

the flavonoids and DNA radicals. The reaction of •OH radicals with DNA produces a, mainly featureless, weakly absorbing, spectrum in the 300–600 nm region, Figure 6, whereas reaction with the flavonoids produces significantly larger spectral changes. The spectra of luteolin radicals, for example, produced upon reaction of luteolin with •OH radicals and also following one-electron oxidation by radiolytically produced selenite and deoxyguanosine radicals ($SeO_3^{\bullet -}$, $dG^{+\bullet}$) exhibit similar features in absorbing less than the parent compound in the 360 nm region and more in the 460 nm region, Figure 6. The differences between the DNA and flavonoid radical spectra enable electron transfer from the flavonoids to the DNA radicals (possibly H• atom transfer from F to DNA radicals) to be followed kinetically. A high concentration of DNA (2 mM in base pairs) is used relative to the flavonoids (10–100 µM) to ensure that the •OH radicals react preferentially with the DNA and, by choosing appropriate wavelengths, the reaction between each flavonoid and the DNA radicals is followed.

$$DNA^{+\bullet} + F \rightarrow DNA + F^{+\bullet} \quad (9)$$

One-electron oxidation of the flavonoids by the DNA radicals was observed both in the presence and absence of O_2. A fast phase, occurring over the first 500 µs is observed both in the presence and absence of O_2, and a slower phase only in the presence of O_2, presumably from peroxyl-type radicals formed on the reaction of O_2 with a proportion of the DNA radicals.

$$DNA^{+\bullet}\ (DNA^{\bullet}/H_3O^+) + O_2 \rightarrow DNAOO^{\bullet} \quad (10)$$

$$DNAOO^{\bullet} + F + H_3O^+ \rightarrow DNAOOH + F^{+\bullet} + H_2O \quad (11)$$

The rates of electron transfer from the flavonoids to the DNA radicals in solutions containing 40 µM of each flavonoid are given in Table I for comparison purposes. The percentage of electron transfer from each flavonoid to the DNA radicals relative to direct oxidation by the $SeO_3^{\bullet -}$ or $dG^{+\bullet}$ radicals

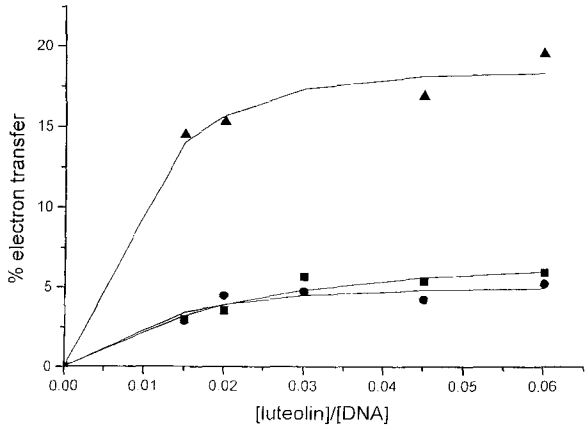

FIGURE 7 Percentage electron transfer from luteolin to $DNA^{+\bullet}$ radicals following pulse radiolysis of solutions (3 Gy in 200 ns) containing DNA (2 mM, base pairs), phosphate buffer (1 mM, pH 7.4) and increasing concentrations of luteolin. (i) Fast phase in N_2O-saturated solution (< 1000 μs), ●; (ii) fast phase in N_2O/O_2-saturated solution (< 1000 μs), ■; and (iii) slow phase in N_2O/O_2-saturated solution (< 50 ms), ▲. Percentage electron transfer is calculated from the yield of luteolin radicals formed upon oxidation by the selenite.

(in the absence of DNA) was determined for increasing concentrations of the flavonoids at a fixed DNA concentration of 2 mM, e.g., for luteolin, Figure 7. Plateau values in the percentage of electron transfer from the flavonoids are reached under all test conditions (N_2O, N_2O/O_2 both fast and slow phases) and the values for each flavonoid are presented in Table I. In the presence of O_2 the yields of the flavonoid radicals associated with the fast phase decrease whereas the combined yields of the fast and slow phases are maintained or increased over the yields measured in the absence of O_2, Table I.

The ease at which compounds undergo oxidation has long been recognised as an important controlling parameter for compounds to act as antioxidants.[51] The one-electron reduction potential, $E(1)$ of individual antioxidant radicals ($A^{+\bullet}/A$) provides a scale by which to compare antioxidants. Literature values, using the pulse radiolysis method, for $E(1)$ for catechin (and hence epicatechin) and quercetin are 0.57 and 0.60 V respectively.[40] We have confirmed the $E(1)$ for catechin (using the Trolox radical as reference[40]) and determined $E(1)$ for luteolin as 0.72 ± 0.01 V at pH 7 using both *p*-phenylenediamine radical ($E(1) = 0.73$ V[39]) and *p*-methoxyphenoxyl radical ($E(1) = 0.73$ V[52]) as reference radicals. Redox equilibrium could not be achieved between apigenin and the *p*-phenylenediamine radical, implying that $E(1)$ for apigenin is > 0.85 V, however redox equilibrium was achieved against kaempferol ($E(1) = 0.95$ V[40]) yielding a value of 1.04 V. Given that the major determinant of $E(1)$ for the flavonoids is the number of hydroxy groups on the so-called B ring, both kaempferol and apigenin are expected to be outliers from the other compounds which span a relatively small range in $E(1)$ (0.57–0.72 V). Our data also shows that 3-hydroxyl substitution of the flavones luteolin and apigenin, to form the flavonols quercetin and kaempferol, has the effect of lowering $E(1)$ values by *ca.* 0.1 V.

DISCUSSION

The results of this *in vitro* study show that dietary flavonoids protect DNA to some degree from free radical damage which can lead to DNA strand breaks and residual base damage, by a mechanism other than solely by direct scavenging of $^{\bullet}OH$ radicals before they react with DNA. Flavonoids are known to scavenge $O_2^{\bullet-}$, e.g. the rate constant for the reaction of $O_2^{\bullet-}$ with catechin has been measured as $6.6 + 0.6 \times 10^4\ M^{-1}\ s^{-1}$.[40] This rate constant is far too low to account for our pulse radiolysis observations and such scavenging is unlikely to play a role in reducing DNA damage since the flavonoids are observed to exert their protective effect both in aerated and hypoxic solutions, which implies that certain lesions on the DNA are unaltered by oxygen and remain capable of oxidising flavonoids. The fact that the dose response curve for strand breaks in the presence of a large excess of carrier DNA is similar to controls indicates that in our system $O_2^{\bullet-}$ is not

contributing to the level of observed strand breaks. Also it is known that $O_2^{\bullet-}$ does not damage DNA to any significant extent[53] and the reaction between $O_2^{\bullet-}$ and certain purine radicals can lead to restitution of the base[54] which might partially explain why there is less sustained damage to DNA in aerobic solution compared to that in hypoxia. Inhibition of DNA strand breaks by minor-groove binding bibenzimidazoles (e.g. Hoechst 33358)[55,56] has been associated with electron transfer from the bound ligands to DNA radicals[57,58] and it is possible that a similar electron transfer mechanism is operating between the flavonoids and the DNA radicals. However, unlike the bibenzimidazoles, which bind tightly to DNA limiting their penetration through tissue, flavonoids such as quercetin are known to bind only weakly to DNA.[59,60] Targeting of flavonoids and other antioxidants that have favourable (lower) one-electron reduction potentials, $E(1)$, for their radical forms, compared to relatively long-lived DNA radicals of higher $E(1)$, may assist the process of electron transfer by effectively increasing the concentration of the antioxidant in the vicinity of the DNA radicals. Our pulse radiolysis results give support for such an electron transfer mechanism taking place from the flavonoids to DNA radicals formed upon $^{\bullet}OH$ radical attack. Possible explanations for the observed decrease in the incidence of strand breaks in aerobic solution compared to that in hypoxia is that the formation of DNA peroxyl radicals, or the reduction of peroxyl radicals to hydroperoxides by the electron transfer, are relatively protective against strand breaks. The efficiency of this electron transfer process, in terms of rate and percentage transfer versus $E(1)$, cannot be fully gauged as, except for apigenin and kaempferol which possess $E(1)$ values of *ca.* ≥ 0.95 V, all of the flavonoids tested have similar $E(1)$ values near 0.6 V. However, for kaempferol and apigenin, there is a significant decrease in both the DMF_{max} and electron transfer rates. While our data gives an indication that $E(1)$ is an important determinant in the effectiveness of the flavonoids in reducing the frequency of DNA strand breaks through an electron transfer mechanism, more work is required, especially with compounds spanning a wider range in $E(1)$ values as well as the rutin and glucosidic forms of the flavonoids.

Our irradiation test conditions of TRIS buffer and aerated solutions produces more endoIII- than Fpg-sensitive sites on the plasmid which is the opposite of that reported for a similar DNA-test system under the conditions of oxygen saturation, phosphate buffer (15 mM) and high salt concentration.[44] On calculating the DMF values for each of the conditions in Figure 5 it is seen that catechin is more effective on purine radicals (1.94) and the precursor radicals to prompt single-strand breaks (1.88) than on pyrimidine radicals (1.69) and the radical precursors to AP site formation (1.67). Some of the decrease in the G (ess) yield seen following Fpg and endoIII treatments of plasmid, irradiated in the presence of catechin, might well be accounted for by the known additional endonuclease activity of Fpg and endoIII for regular AP sites. A slightly greater efficiency in the repair of pyrimidine radicals by the flavonoids through electron transfer relative to purine radicals might be expected in light of the known chemistry of the $^{\bullet}OH$ radical adducts of the four DNA bases.[28] On one-electron reduction of the $^{\bullet}OH$ radical adducts of both cytosine and thymine water is expected to be eliminated to give back the parent compounds. However the chemistry of the various $^{\bullet}OH$ radical adducts of adenine and guanine is more complex. Some adducts undergo ring-opening reactions, and thus will not be repaired on one-electron reduction, and some one-electron reduced adducts also lead to new products via ring opening. A possible interpretation of our data is that catechin is more effective on purine radicals than on pyrimidine radicals in decreasing the amount of damage or (more likely) that some of the pyrimidine radicals undergo rapid electron transfer to form purine radicals.[32]

The combination of pulse radiolysis investigations with DNA strand break and base damage measurements described in this study might

serve as a useful tool to rank the effectiveness of different classes of phytochemicals in regard to their antioxidant activity against DNA radicals. Such a ranking would aid the interpretation of epidemiologic studies on possible relationships between dietary antioxidants and the prevention of disease. The comet assay has been used to study the effect of vitamin C on oxidative damage to DNA,[61] however it is difficult to make a distinction between free radical scavenging and repair of radical damage on the DNA. Assays have been developed which determine the relative antioxidant activity of solutions based on the scavenging of coloured radical cations, e.g. the "TEAC" assay using the antioxidant Trolox as a reference.[62] While such assays are useful in evaluating compounds in terms of them undergoing electron transfer reactions, no precise information on how test compounds interact with DNA radicals is possible. Others have also pointed out the need for radical assays of antioxidant activity to be relevant to oxidisable substrates in biological systems.[63,64] Intervention studies have been mounted using vitamin C, vitamin E and β-carotene on the basis that these substances are well-known nutrients that are antioxidants. Not only did a study on the effects of vitamin E and β-carotene supplementation, to possibly reduce the incidence of lung and other cancers in smokers, show no benefit, but potential harm was indicated on β-carotene supplementation.[65] Our electron transfer studies may well be of particular relevance to past and future epidemiologic studies in regard to the relationship between the consumption of fruits and vegetables, as well as dietary supplements, and some protection against a range of cancers. Preliminary studies indicate that the water soluble form of vitamin E (Trolox) is mainly ineffective in repairing DNA radicals and vitamin C shows a much lower efficiency than all of the flavonoids tested in this study. (R.F. Anderson and L.J. Fisher, unpublished work).

Although *in vitro* studies give some insight into mechanisms by which the flavonoids can act as antioxidants certain flavonoids are readily autoxidised in chemical systems which may explain why only low levels of some flavonoids, e.g. quercetin, are found in human plasma even following supplementation.[66,67] Fuller understanding of the possible health benefits of the flavonoids awaits further information on their absorption, distribution and metabolism in humans, studies which are being aided by improved analytical methods.[68,69] Our results support the possibility that the flavonoids can perform an additional role to that of simple free radical scavenging, namely reducing the amount of free radical lesions on DNA. However, our studies do not directly address the question if fast chemical repair of DNA damage through electron transfer occurs in mammalian DNA *in vivo*. While such an electron transfer mechanism has been shown to occur between certain antioxidants and deoxyguanosine radicals[30,37] the method reported in this paper enables quantification of such a reaction between DNA radicals and antioxidants.

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