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Effects of Dietary Antioxidants on Human DNA Ex Vivo

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The protective effect of fruits and vegetables against cancer is well established. It is believed that this effect is mediated by antioxidants and decreased oxidative damage to DNA. However, the identity of the antioxidant(s) responsible is not clear. Moreover, a potentially damaging pro-oxidant effect of some antioxidants has been reported. In this study the *ex vivo* effects of several dietary antioxidants, including quercetin, various catechins, ascorbic acid and α-tocopherol, were investigated, at concentrations up to 200 μM, using the single cell gel electrophoresis (comet) assay for DNA damage. Lymphocytes from three healthy subjects were pre-incubated with these antioxidants, and the comet assay was performed on treated, untreated, challenged and unchallenged cells in parallel, oxidant challenge being induced by 5 min exposure to hydrogen peroxide (final concentrations H_2O_2: 30, 45, or 60 μM). Results using this *ex vivo* cellular assay showed protection by some antioxidants (quercetin, caffeic acid), no effect by some (catechin, epicatechin, catechin gallate, epicatechin gallate) and an apparently damaging effect by others (epigallocatechin, epigallocatechin gallate). Damage may have been caused by production of H_2O_2 from these polyphenolics. Neither ascorbic acid nor α-tocopherol protected or damaged DNA. Further study of the role of quercetin and caffeic acid in DNA protection is needed.

Keywords: Antioxidant; Comet assay; Quercetin; Caffeic acid; DNA; Oxidative stress

INTRODUCTION

Epidemiological studies have shown that high intake of antioxidant-containing foods is associated with lower risk of chronic disease.[1–5] Oxidative stress is associated with DNA damage, lipid peroxidation and protein cross-linking and, via oxidative changes to key biomolecules, is believed to increase risk of cancer, coronary heart disease, cataract and dementia, and to be deeply involved in the ageing process.[1–6] Antioxidants may ameliorate this, however cause and effect relationships have not yet been established. Intervention trials with antioxidants have not shown clearly protective effects to date,[7–12] and a pro-oxidant effect has been reported for some antioxidants, including ascorbic acid.[13–17]

Conceptually, if DNA damage increases risk of cancer, then an agent which protects DNA against damage will protect against cancer. A useful method of assessing DNA damage is the single cell gel electrophoresis, or "comet", assay, which detects DNA strand breaks in individual cells.[18] In brief, loops of DNA containing single- or double-stranded breaks are pulled out of the nucleus of lysed cells by an electric field, thus forming a "comet" tail. The amount of DNA in the tail is related to the degree of DNA damage incurred.[18,19] This assay is useful, as small numbers of cells can be used, it is relatively quick, standardized conditions can be used, and different test agents can be run in parallel, allowing direct comparison of *ex vivo* effects, and the comet assay has been used successfully in biomonitoring and antioxidant studies.[19–23] The aim of this current study was to use this cellular assay to assess *ex vivo* DNA protective or damaging effects of selected dietary antioxidants, including ascorbic acid, α-tocopherol, quercetin and various catechins.

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MATERIALS AND METHODS

Purest or molecular biology grade of the following was purchased: RPMI 1640 and fetal bovine serum were from GibcoBRL, Paisley, UK; Histopaque 1077 and dimethyl sulfoxide (DMSO) were from Sigma, St Louis, MO, USA; type VII low gelling point agarose, standard agarose, phosphate buffered saline (PBS) tablets, sodium chloride, disodium ethylenediaminetetraacetic acid dihydrate, hydrogen peroxide solution, hydrochloric acid, Tris[hydroxymethyl]aminomethane, ethidium bromide, Triton X-100, quercetin, caffeic acid, catechin, epicatechin, catechin gallate, epigallocatechin gallate, epigallocatechin and epicatechin gallate were from Sigma; L-(+)-ascorbic acid and DL-α-tocopherol were from Merck, Darmstadt, Germany; sodium hydroxide was from Riedel-de Haen, Garmany.

For the purified polyphenols, 1.0 mM stock solutions were prepared in PBS (assisted by sonication) and further diluted in PBS before testing. Solutions of ascorbic acid were prepared in PBS and used within 1 h. Working solutions of α-tocopherol were prepared in PBS from 10 mM α-tocopherol stock solution in absolute ethanol on the day of testing; final ethanol concentration was less than 1%.

Lymphocytes from three healthy, consenting subjects (one male, two females) were harvested from venous blood following the procedure of Collins and co-workers.[19] Forty μl of blood (containing approximately 4×10^4 lymphocytes) were added to 1 ml chilled RPMI medium containing 10% fetal bovine serum (FBS) in a 1.5 ml microcentrifuge tube. Blood and medium were mixed gently and left on ice for 30 min before underlaying with 100 μl Histopaque 1077. Tubes were spun at 2000 rpm for 5 min at 4°C. Lymphocytes were retrieved in 100 μl from just above the boundary between the RPMI and Histopaque layers, and added to 1 ml chilled PBS in a microcentrifuge tube. The centrifugation step was repeated and as much supernatant as possible was removed from the pelleted lymphocytes. Cells from each individual were tested, in separate experiments, with each antioxidant test agent immediately after harvesting or within one month of cryopreservation. Cryopreservation does not affect baseline DNA damage or the response to oxidant stress, and was performed on the day of blood collection and according to the validated procedure of Collins *et al.*[19]

For antioxidant treatment and oxidant challenge of lymphocytes, 1 ml of PBS (with or without antioxidant test agent at between 12.5 and 200 μM) was added to the tube containing washed, pelleted lymphocytes and gently mixed. The cell suspension was incubated at 37°C for 30 min. The tubes were then centrifuged, the supernatant was discarded, cells were washed once with 1 ml cold PBS and the centrifugation step was repeated. One ml of freshly prepared H_2O_2 (final concentrations: 0, 30, 45 and 60 μM) in PBS was then added to the pelleted lymphocytes to induce oxidative stress. The oxidant/cell mixtures were kept on ice for 5 min, then centrifuged as before. Cells were washed with 1 ml cold PBS and centrifugation repeated. As much supernatant as possible was removed, and the comet assay was performed on the treated, untreated, challenged and unchallenged cells in parallel. Cell viability was assessed using the trypan blue exclusion test[24] before and after incubation with testing agents and H_2O_2. Results showed >95% cell survival in all cases.

The comet assay was performed following the procedure of Collins and co-workers.[19] Lymphocytes (treated, untreated, challenged and unchallenged) were mixed with 85 μl of pre-warmed (40°C) 1% (w/v) low gelling point agarose in PBS, and immediately applied to a microscope slide which had been pre-coated with 1% (w/v) standard agarose in PBS. The slides were placed at 4°C until the gel layer solidified, after which slides were submerged in lysis solution and in the dark for 1 h at 4°C. Lysis solution comprised 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris (adjusted to pH 10 by adding concentrated or solid NaOH); 500 μl of Triton X-100 were added to 50 ml of this solution just before use. After lysis, slides were transferred to an electrophoresis tank (Sub-Cell GT, Bio-Rad, CA, USA) containing 300 mM NaOH and 1 mM EDTA electrophoresis solution, ensuring that the slides were submerged. DNA unwinding and expression of alkali-labile sites were allowed to proceed for 40 min. Electrophoresis was then performed for 30 min at 25 V constant voltage. The current was adjusted to 0.30 A by adjusting the level of the electrophoresis solution in the tank. After electrophoresis, slides were removed and neutralized by immersion in three changes (3× 5 min) of 0.4 M Tris at pH 7.5. Slides were stained with ethidium bromide (20 μg/ml); image analysis proceeded without delay after the staining of each individual slide.

Fifty cells were scored per treatment in each of three independent series of experiments, each series using cells from a different subject, i.e. 150 cells for each antioxidant at each dose tested, and at each H_2O_2 concentration were scored. Analysis of comets was performed using a fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan, fitted with a 580 nm emission filter) by measuring the tail DNA content, which is a commonly used index of DNA damage in this assay.[19,20] The DNA content (%) measures the intensity of light of the comet tail, and is an index of the proportion of DNA migrating into the tail. Computerized image analysis system was used for tail DNA content measurement (Komet 3.0,

Kinetic Imaging, Liverpool, UK). Dunnett's test was used to investigate differences in tail DNA content between treated and untreated, challenged and unchallenged cells.

Ethical approval for this study was granted by the Ethics Subcommittee of The Hong Kong Polytechnic University, and all procedures involving human subjects complied with the Declaration of Helsinki, as revised in 1996.

RESULTS

DNA damage clearly increased with oxidant challenge induced by H_2O_2. Pre-treatment with quercetin and caffeic acid induced no DNA damage *per se* and increased DNA resistance to oxidant challenge (Figs. 1 and 2). No damaging effect was seen with increasing concentrations of quercetin or caffeic acid in unchallenged cells.

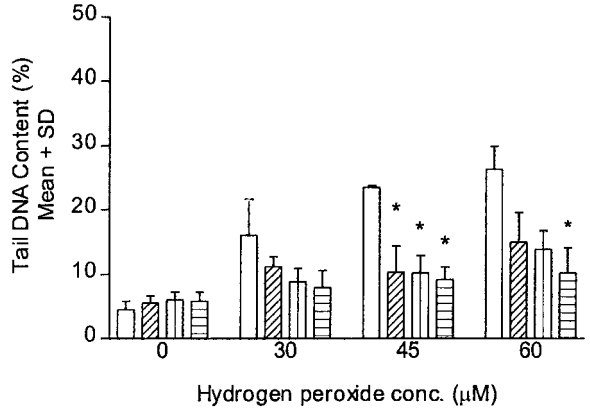

FIGURE 1 Effect of quercetin on tail percent DNA content (mean+1SD) of lymphocytes from three healthy subjects tested in separate experiments. Results on challenged (with H_2O_2) and unchallenged cells (no H_2O_2) pre-incubated with quercetin at 0 μM (open bars), 12.5 μM (diagonal bars), 25 μM (vertical bars), 50 μM (horizontal bars) are shown. ($*P < 0.05$).

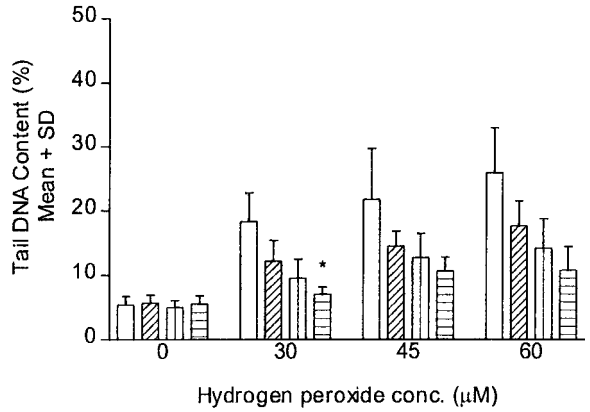

FIGURE 2 Effect of caffeic acid on tail percent DNA content (mean+1SD) of lymphocytes from three healthy subjects tested in separate experiments. Results on challenged (with H_2O_2) and unchallenged cells (no H_2O_2) pre-incubated with caffeic acid at 0 μM (open bars), 25 μM (diagonal bars), 50 μM (vertical bars), 100 μM (horizontal bars) are shown ($*P < 0.05$).

H_2O_2 clearly caused increased damage in challenged cells without pre-treatment. Decreased DNA damage was seen in cells pre-treated with quercetin or caffeic acid. Pre-treatment with epigallocatechin gallate and with epigallocatechin induced DNA damage without additional H_2O_2--induced oxidant stress (Fig. 3a, b). Pre-treatment with catechin, catechin gallate, epicatechin and epicatechin gallate did not damage DNA, but no DNA protection was seen in pre-treated cells exposed to H_2O_2 challenge (results not shown). No damaging effect was seen with increasing concentrations of ascorbic acid or α-tocopherol. No protective effect was seen with ascorbic acid. There was some evidence of protection by α-tocopherol in each of the individual experiments, but no significant overall protective effect was seen (Fig. 4a, b).

DISCUSSION

Antioxidants which oppose oxidative damage to DNA, it is hypothesised, decrease risk of cancer.[1–5]

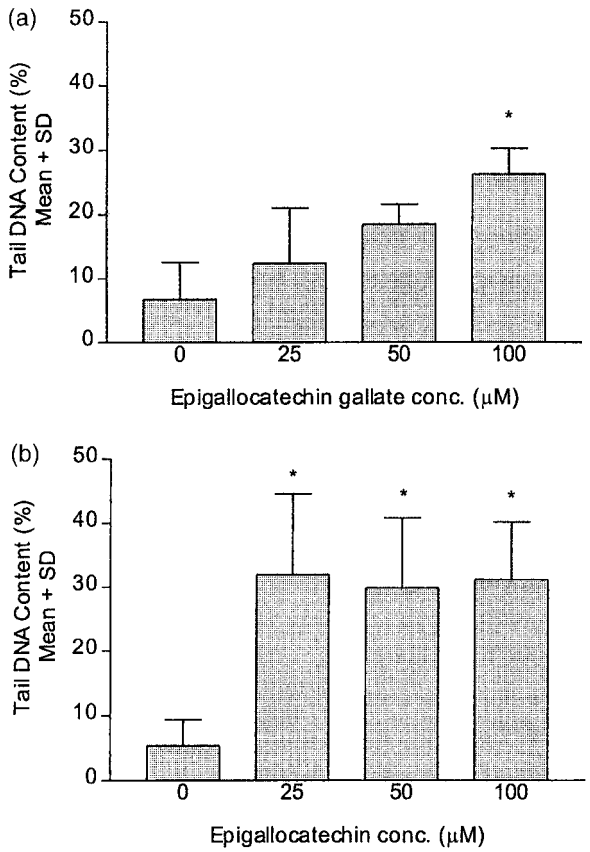

FIGURE 3 Effect of epigallocatechin gallate and epigallocatechin on tail percent DNA content (mean+1SD) of lymphocytes from three healthy subjects tested in separate experiments. Cells pre-treated with (a) epigallocatechin gallate, (b) epigallocatechin, but not exposed to H_2O_2, showed evidence of increased of DNA damage. Damage was more marked with epigallocatechin pretreatment ($*P < 0.05$).

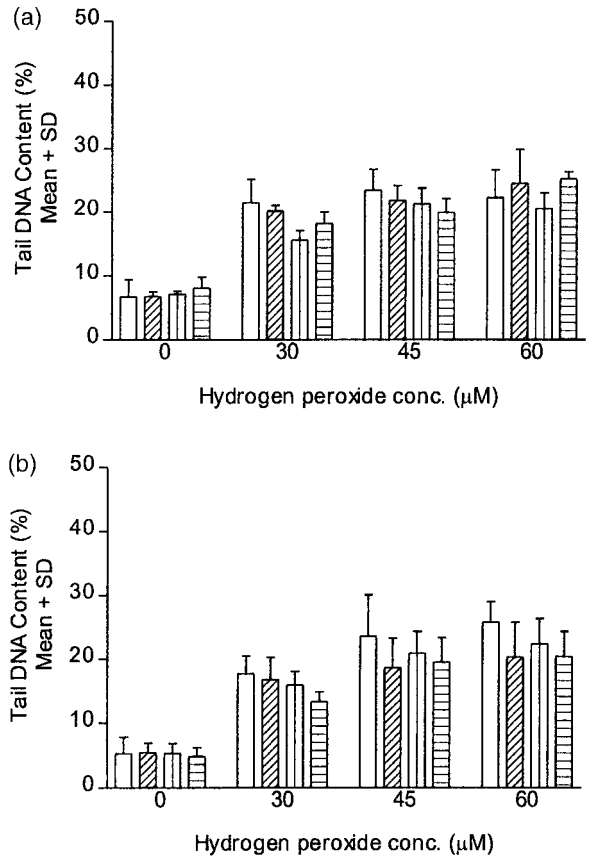

FIGURE 4 Effect of ascorbic acid and α-tocopherol on tail %DNA content (mean+1SD) of lymphocytes from three healthy subjects tested in separate experiments. Results on challenged (with H_2O_2) and unchallenged cells (no H_2O_2) pre-incubated with (a) ascorbic acid at 0 μM (open bars), 50 μM (diagonal bars), 100 μM (vertical bars), 200 μM (horizontal bars) and (b) α-tocopherol at 0 μM (open bars), 25 μM (diagonal bars), 50 μM (vertical bars), 100 μM (horizontal bars) are shown.

Diets rich in fruits and vegetables are clearly protective, however supplementation studies with ascorbic acid and α-tocopherol, prime candidates as key protective agents, have shown no clear evidence of benefit, and have been reported to cause DNA damage in some studies.[7–16,25] In this study, ascorbic acid and α-tocopherol showed no convincing DNA protective effect, but neither did they induce DNA damage at the concentrations tested (up to 100 μM for α-tocopherol, up to 200 μM for ascorbic acid). Most of the antioxidant content of fruits and vegetables is not due to ascorbic acid and α-tocopherol, however, but to a multitude of phenolic phytochemicals,[26–29] and DNA protection of diets rich in fruits and vegetables may be mediated by one or more of these antioxidants, rather than vitamins C and E. Results of this study indicate that caffeic acid, a hydroxycinnamic acid found in coffee, berries, apples and pears, and quercetin, a flavonoid found in tea, apples, spinach and onions,[29,30] protect DNA against oxidant challenge in an *ex vivo*, whole cell model. The other polyphenolic antioxidants tested were not protective, and some, namely epigallocatechin gallate and epigallocatechin, induced DNA damage.

There has been no previous report of the effect of caffeic acid using the comet assay. Caffeic acid has been shown to be almost completely absorbed from the gastrointestinal tract, but plasma levels are unknown.[31] In this current study, a protective effect was seen at 12.5 μM quercetin, which supports previous studies investigating the effect of quercetin on human lymphocyte DNA.[20,21] The study by Duthie *et al.*[20] showed protection at quercetin concentrations >10 μM. Noroozi *et al.*[21] calculated that 47 μM quercetin would reduce DNA damage by 50%. This concentration is likely to be found in the gastrointestinal tract after ingestion of quercetin-rich foods and beverages, but plasma levels of quercetin are usually <4 μM.[32,33] However, a recent study[34] reported that 3 μM quercetin protected the crystalline lens from H_2O_2-induced challenge. Quercetin at physiological concentrations, therefore, may confer significant protection against intracellular oxidant challenge.

During preparation of this paper, a report was published[35] on the effects of quercetin and epigallocatechin on damage to DNA in cultured Jurkat T-lymphocytes. Damage was assessed, as in our study, using the comet assay and a 30 min pre-treatment time. Quercetin at 10 μM protected Jurkat T lymphocyte DNA against 25 μM H_2O_2. However, it was also reported[35] that 10 μM epigallochatechin gallate was protective, and that quercetin and epigallocatechin gallate at 100 μM damaged DNA. In this current study, no DNA damaging effect was seen at up to 50 μM quercetin, the highest dose tested, and epigallocatechin gallate showed no protection. Furthermore, our results indicated a dose-related increase in DNA damage with epigallocatechin gallate. This current study also showed that epigallocatechin induced significant DNA damage. It is of interest here that catechins have been reported[36] recently to generate H_2O_2 in cell culture media, with around 70 and 100 μM H_2O_2 formed after 60 min from 100 μM of, respectively, epigallocatechin gallate and epigallocatechin. No H_2O_2 was reportedly generated by these polyphenolics in solutions prepared in distilled water, however generation of H_2O_2 by polyphenolic test agents may have occurred in our cellular test system during the 30 min pre-incubation time, imposing an "antioxidant-induced" oxidant stress. The DNA damage seen in cells pre-incubated with 100 μM of epigallocatechin in this study was greater than that in cells pre-incubated with the same concentration of epigallocatechin gallate. This is in agreement with their apparent H_2O_2 generating power,[36] and was similar to the damage seen in cells exposed to 30–45 μM H_2O_2. Generation of H_2O_2 by epigallocatechin

gallate could also account for the damaging effect reported by Johnson and Loo.[35] Catechin and quercetin were reported[36] to generate much smaller amounts of H_2O_2, and this is in agreement with the lack of any significant DNA damage seen in our cells pre-incubated with these agents. Nevertheless, very high concentrations of these may also generate enough H_2O_2 to damage DNA. This could be the explanation of the damage reportedly seen at 100 μM quercetin.[35]

The mechanism by which quercetin protects DNA is not clear. Quercetin is a powerful antioxidant with metal-ion binding properties, as well as radical scavenging abilities. However, catechins also have these antioxidant properties and were found to confer no DNA protection. Indeed, some catechins induced damage. Furthermore, ascorbic acid is also a scavenging antioxidant, but showed no protective effect in this current study. This implies that the protective effect of quercetin is mediated by a non-scavenging mechanism. Quercetin is reported to gain access to cells[20,34] and may, by binding tightly to intracellular iron and copper, prevent hydroxyl radical formation, or at least prevent its generation in the immediate vicinity of DNA bases. It is possible that quercetin is a more effective chelator than catechins. Quercetin has been reported to bind iron, copper and manganese, while catechins reportedly bind only copper.[35] Prevention of copper- and iron-mediated hydroxyl radical formation, combined with a lower peroxide generating power, may make quercetin much more effective than catechins in terms of protection against oxidative damage. It is possible also that there may be a protective adaptation of cells in response to a challenge from low levels of quercetin-generated H_2O_2. Further study, using catalase, is now underway to determine if the protective effect of quercetin and caffeic acid, and the damaging effects of some of the catechins tested, is mediated by H_2O_2.

In this current study, ascorbic acid at levels representative of plasma (up to 200 μM) showed no DNA protective influence, but no ascorbic acid-induced DNA damage was seen. This is of interest because ascorbic acid has been reported to increase oxidative DNA-base damage in lymphocytes[12,15,17] and to cause strand breaks.[37] However, use of vitamin C supplements is very common, and there is no evidence to suggest that healthy subjects with high intake of ascorbic acid are at increased risk of cancer. It is unlikely that ascorbic acid induces DNA damage under normal circumstances, but it is possible that ascorbic acid is not directly genoprotective, and that a pro-oxidant effect may be manifest under certain intracellular conditions or concentrations. It must be noted that the lack of a DNA-damaging effect of vitamin C is not conclusive, as strand breakage is not the only kind of DNA damage which can be induced, and the concentration of ascorbic acid tested reflected plasma, rather than intracellular, levels. An enzyme-assisted comet assay[20] can be used to reveal, specifically, oxidised DNA lesions, and it would be interesting to use this modified version to determine if ascorbic acid, and perhaps quercetin and catechins, induce base oxidation at concentrations where no overt strand breakage occurs.

In this current study, α-tocopherol showed no pro-oxidant effect, but no significant protection was seen. This is in agreement with data from a similar study,[20] however some slight protective effect was seen in challenged cells from each of our three subjects in the individual experiments. Previously reported data from a lymphoblastoid cell line and using 24 h pre-incubation with α-tocopherol,[22] and from human supplementation trials[11,23] indicate that α-tocopherol protects DNA against oxidant damage. It may be that a longer incubation time than the 30 min used in this current study is needed for cellular uptake of α-tocopherol. Alpha-tocopherol is a lipid-soluble antioxidant found mainly in cell membranes, and its role in DNA protection is likely to be an indirect one. For example, by interacting with ROS within the cell membrane α-tocopherol decreases the oxidant load reaching the nucleus, and enhanced antioxidant protection of polyunsaturated fatty acids decreases DNA exposure to mutagenic and carcinogenic lipoperoxide degradation products such as malondialdehyde.[38]

In conclusion, our results of parallel testing using an *ex vivo* cellular assay indicate that DNA in human lymphocytes is damaged by some dietary antioxidants, protected from oxidant challenge by others, and that some dietary antioxidants, including ascorbic acid, have no discernable effect. The mechanism of damage is not clear, but could be related to H_2O_2 production by the supposed antioxidant. The mechanism of protection exhibited by quercetin and caffeic acid may be direct ROS scavenging, however, as no protection was seen with ascorbic acid, an effective scavenger, it is likely to involve another process, such as iron binding, or possibly, an adaptive response to a low level of oxidant challenge induced by the "antioxidant". These results are useful, along with other evidence from *in vivo* and *in vitro* studies of antioxidant and proxidant activity of micronutrients, but further work is needed to clarify behaviour at different concentrations and the mechanism of action.

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