Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA

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Abstract:

Phenolic phytochemicals are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against free radicals. The aims of this study were to assess the free radical-scavenging activities of several common phenolic phytochemicals, and then, the effects of the most potent phenolic phytochemicals on oxidative damage to DNA in cultured cells. Epigallocatechin gallate (EGCG) scavenged the stable free radical, α,α-diphenyl-β-picrylhydrazyl (DPPH), most effectively, while quercetin was about half as effective. Genistein, daidzein, hesperetin, and naringenin did not scavenge DPPH appreciably. Jurkat T-lymphocytes that were pre-incubated with relatively low concentrations of either EGCG or quercetin were less susceptible to DNA damage induced by either a reactive oxygen species or a reactive nitrogen species, as evaluated by the comet assay. More specifically, control cells had a comet score of only 17 ± 5, indicating minimal DNA damage. Cells challenged with 25 pM hydrogen peroxide (H_2O_2) or 100 μM 3-morpholinosydnonimine (SIN-1, a peroxynitrite generator) had comet scores of 188 ± 6 and 125 ± 12, respectively, indicating extensive DNA damage. The H_2O_2-induced DNA damage was inhibited with 10 μM of either EGCG (comet score: 113 ± 23) or quercetin (comet score: 82 ± 7), Similarly, the SIN-1-mediated DNA damage was inhibited with 10 μM of either EGCG (comet score: 79 ± 13) or quercetin (comet score: 72 ± 17), In contrast, noticeable DNA damage was induced in Jurkat T-lymphocytes by incubating with 10-fold higher concentrations (i.e., 100 pM) of either EGCG (comet score: 56 ± 17) or quercetin (comet score: 64 ± 13) by themselves. Collectively, these data suggest that low concentrations of EGCG and quercetin scavenged free radicals, thereby inhibiting oxidative damage to cellular DNA. But, high concentrations of either EGCG or quercetin alone induced cellular DNA damage.

Keywords:

Antioxidants; DNA damage; Free radicals; Phenolic phytochemicals

Article:

1. INTRODUCTION

Cells must maintain a proper balance between the levels of free radicals and antioxidants to ensure the structural integrity of critical components. When the levels of free radicals exceed that of antioxidants during oxidative stress, sensitive biomolecules such as lipids, proteins and DNA in particular can be damaged. As a result, numerous degenerative chronic diseases may develop [1].

Reactive oxygen species (e.g., hydrogen peroxide or H_2O_2) and reactive nitrogen species (e.g., peroxynitrite) can especially be destructive when present in excess. They may cause irreparable DNA damage, leading to mutagenesis and perhaps cancer [2]. Thus, there is deep interest in identifying free radical scavengers or antioxidants that inhibit oxidative DNA damage. Accordingly, phenolic phytochemicals, which are present in many edible plant products, have been examined in non-cellular models by a number of researchers. For example, the class of phenolic phytochemicals known as flavonoids scavenged nitric oxide [3] and peroxynitrite

[4], as determined by measuring the reduction of nitric oxide concentration with an electrode sensor and the oxidation of dihydrorhodamine 123 with a fluorometer, respectively. When purified calf thymus DNA was exposed to either $FeCl_2/H_2O_2$ or UV radiation that both generate hydroxyl radicals, phenolic phytochemicals inhibited the formation of 8-hydroxy-2'-deoxyguanosine [5]. However, these non-cellular studies [3–5] do not reveal if phenolic phytochemicals have similar scavenging action against oxygen and nitrogen radicals so as to protect DNA in actual cells. This research gap has been previously addressed to some extent. For instance, the phenolic phytochemical, epigallocatechin gallate (EGCG), inhibited phorbol ester-induced production of H_2O_2 and oxidative DNA damage in HeLa cervical carcinoma cells [6]. Such previous cellular findings were expanded by the present study.

Using a rapid screening procedure, we first deter- mined the radical-scavenging activities of six com- mon phenolic phytochemicals (Fig. 1). Using Jurkat T-lymphocytes, we next determined if the most po- tent scavengers inhibit oxidative damage to DNA, which was induced by either H_2O_2 or 3-morpholino-

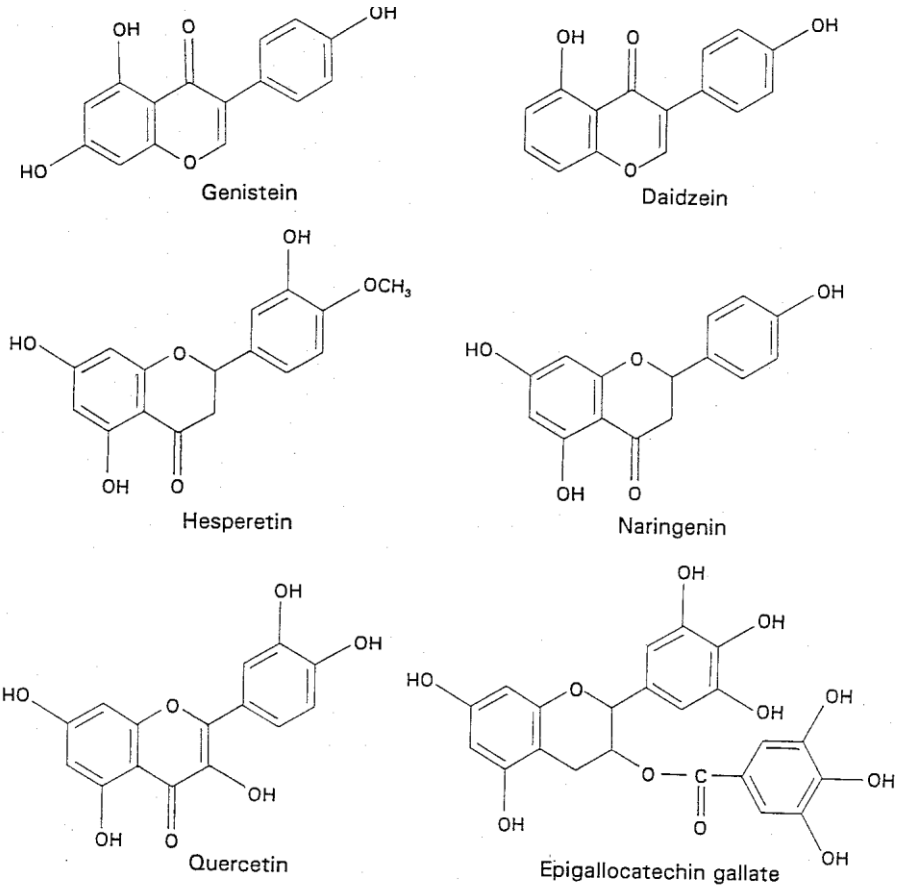

Fig. 1. Structures of the six phenolic phytochemicals initially screened for radical-scavenging activity.

sydnonimine (SIN-1), a peroxynitrite generator [7].

Additionally, because phenolic phytochemicals have been reported to have pro-oxidant effects in non-cellular systems [8,9], we also determined if phenolic phytochemicals by themselves actually induce oxidative DNA damage in Jurkat T-lymphocytes.

2. MATERIALS AND METHODS

2. 1. Materials

Low and normal melting point agarose products were purchased from Sigma (St. Louis, MO). All reagents were purchased from Fisher Scientific (Norcross, GA) or Sigma, unless otherwise stated.

2.2. Initial screening of phenolic phytochemicals for radical-scavenging activity

The radical-scavenging activities of genistein, daidzein, hesperetin, naringenin, quercetin, and EGCG were evaluated by determining their abilities to chemically reduce the stable free radical, α,α-diphenyl-β-picrylhydrazyl (DPPH) [10]. To 1.4 ml of 0.1 μM DPPH (dissolved in ethanol), 0.1 ml of 10 μM phenolic phytochemical was added. After allowing the reaction to take place, the final absorbance was measured at 516 nm on a Beckman DU-640 recording spectrophotometer and compared to the absorbance of DPPH in the absence of phenolic phytochemical.

2.3. Cell culture and treatment

Using a 95% air/5% CO2 humidified incubator set at 37°C, Jurkat T-lymphocytes (ATCC, Rockville, MD) were grown in RPMI 1640 culture media supplemented with 10% FBS, 1% glutamine, 1% penicillin–streptomycin (10,000 units/ml), and 0.2% amphoterecin B (250 μg/ml). Cells (0.5 = 105/ml in a 12-well microplate) were pre-incubated without and with 10 μM of either quercetin or EGCG inside the humidified incubator for 30 min to allow for cellular uptake of the phenolic phytochemicals. Afterwards, cells were washed with ice-cold phosphate-buffered saline (PBS), pH 7.4. Following centrifugation, the cell pellet was resuspended in 1 ml of PBS. Cells were then challenged at 4°C with 25 μM H_2O_2 or at 37°C with 100 μM SIN-1 for 30 min and immediately analyzed for extent of DNA damage. In other experiments, Jurkat T-lymphocytes were incubated with 10-fold higher concentrations (i.e., 100 μM) of the phenolic phytochemicals by themselves to determine any pro-oxidant effects on DNA.

2.4. Assessment of cellular DNA damage

To determine the extent of DNA damage in cells, alkaline micro-gel electrophoresis [11] or the "comet assay"(Fig. 2) was performed under dim light. Briefly, following treatment and washing, cells were suspended in 1% low melting point agarose dissolved in PBS. Then, 75 μl of this mixture was pipetted onto a frosted microscope slide that had been pre-coated with 75 μl of 1% normal melting point agarose. Without delay, a glass cover slip was placed on top of the slide, and the agarose/cell mixture was allowed to completely congeal by putting the slide on a cold metal tray for 10 min. After removing the cover slip, the slide was immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM disodium EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100) for 1 h. Next, slides were placed in a submarine gel electrophoresis unit containing 300 mM NaOH and 1 mM EDTA, pH 13, for 40 min before being electrophoresed at 20 V (300 mA) for 20 min. Following electrophoresis, the slides were immersed in neutralizing buffer (0.4 M Tris–HCl, pH 7.5), before finally applying 60 μl of 2 μg/ml ethidium bromide on them to stain the DNA.

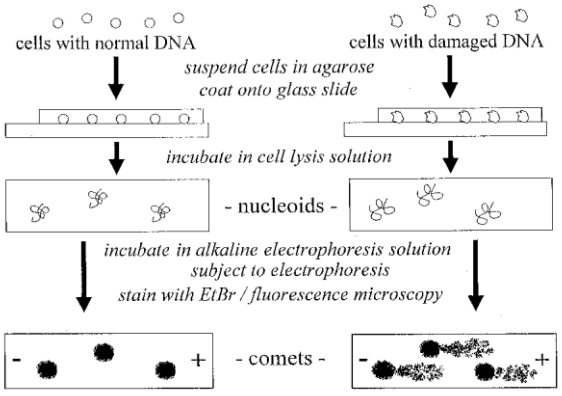

Fig. 2. Principle of alkaline micro-gel electrophoresis (i.e., comet assay) to assess cellular DNA damage.

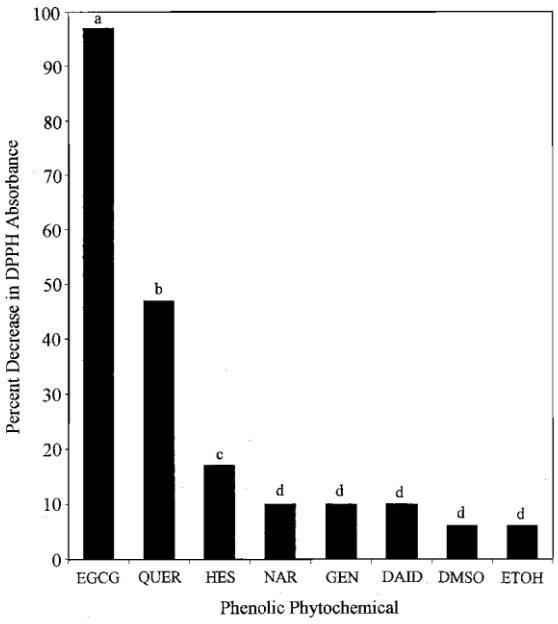

Fig. 3. Spectrophotometric analysis of the phenolic phytochemicals for antioxidant activity. Genistein (GEN), daidzein (DAID), hesperetin (HES), naringenin (NAR), quercetin (QUER), and epigallocatchin gallate (EGCG), as well as their vehicle solvent controls dimethyl sulfoxide (DMSO) and ethanol (ETOH), were reacted with α,α-diphenyl-β-picrylhydrazyl (DPPH). The decrease in absorbance, reflecting chemical reduction of DPPH, was measured at 516 nm. Data represent the mean ± S.E.M. (error bars are not visible due to small values) of three experimental determinations. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

Slides were viewed using an Olympus IX-70a inverted fluorescence microscope. Fifty nucleoids per slide were scored visually for comet tail size based on an arbitrary scale of 0–4, i.e., ranging from no damage to extensive damage of DNA. Individual nucleoid scores were added to give the final comet score reported. This basic procedure of scoring comets compares acceptably with computer imaging analysis [12].

2.5. Statistical treatment of data

Data were analyzed with a standard SAS program using analysis of variance (ANOVA) and Duncan's new multiple range test to determine any significant differences ($P < 0.05$).

3. RESULTS

The antioxidant activities of genistein, daidzein, hesperetin, naringenin, quercetin, and EGCG were first evaluated spectrophotometrically (Fig. 3) by assessing the capacity of each compound to scavenge DPPH, whose absorbance in solution decreases upon accepting hydrogen atoms from an antioxidant [10]. Both EGCG and quercetin effectively scavenged DPPH as indicated by the 97% and 47% decreases in the absorbance of DPPH, respectively. In contrast, hesperetin, genistein, daidzein, and naringenin were weaker scavengers of DPPH, producing only 17%, 10%, 10%, and 10% decreases in DPPH absorbance, respectively. The solvents (i.e., dimethyl sulfoxide and ethanol) for the phenolic phytochemicals did not appreciably decrease DPPH absorbance.

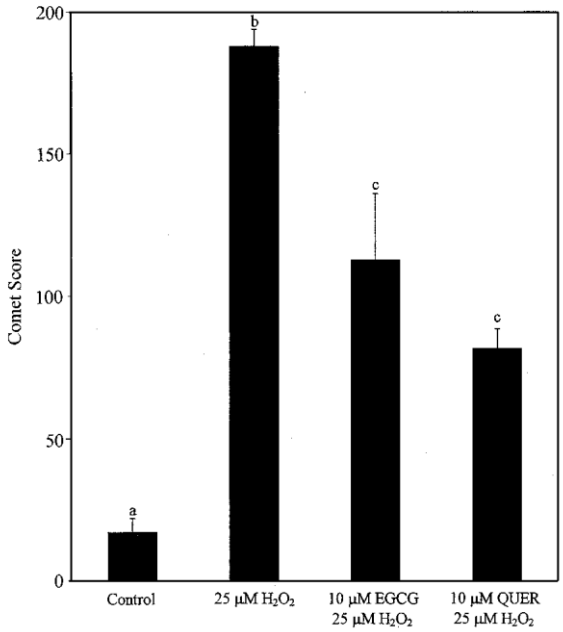

Fig. 4. Inhibitory effect of epigallocatchin gallate (EGCG) and quercetin (QUER) on hydrogen peroxide (H_2O_2)-induced oxidative damage to DNA in Jurkat T-lymphocytes. Cells were pre-incubated with 10 μM of either EGCG or QUER at 37°C for 30 min before being challenged with 25 μM H_2O_2 at 4°C for 30 min. The extent of oxidative damage to DNA was assessed by comet assay, as described in Section 2. Data represent the mean ± S.E.M. of four experimental determinations. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

Having established that EGCG and quercetin were the most potent of the six phenolic phytochemicals tested, we next determined whether each of the two compounds could inhibit oxidative damage to cellular DNA (Fig. 4), as evaluated by the comet assay. Control cells had a comet score of 17 ± 5, whereas cells challenged with 25 mM H_2O_2 had a comet score of 188 ± 6 that indicates extensive DNA damage. However, 10 μM of EGCG and quercetin significantly inhibited H_2O_2-mediated damage to cellular DNA, as indicated by the lower comet scores of 113 ± 23 and 82 ± 7, respectively. Oxidative challenge by 100 μM SIN-1 also induced substantial DNA damage in Jurkat T-lymphocytes, producing a comet score of 125 ± 12 (Fig. 5). Such cellular DNA damage was significantly inhibited by 10 μM of either EGCG (comet score: 79 ± 13) or quercetin (comet score: 72 ± 17).

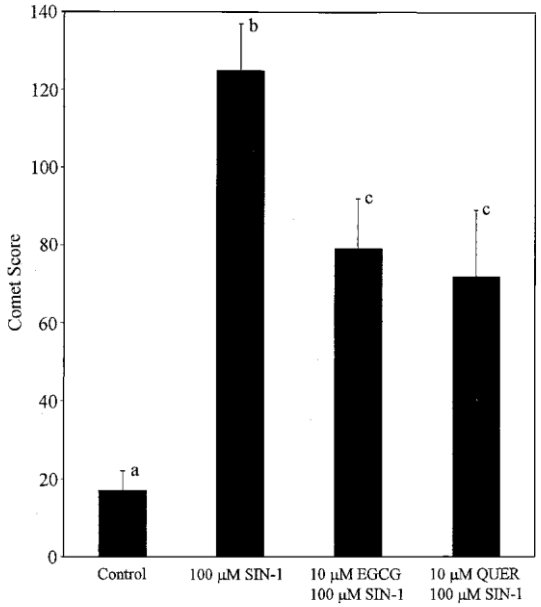

Fig. 5. Inhibitory effect of epigallocatchin gallate (EGCG) and quercetin (QUER) on 3-morpholinosydnonimine (SIN-1)-induced oxidative damage to DNA in Jurkat T-lymphocytes. Cells were pre-incubated with 10 μM of either EGCG or QUER at 37°C for 30 min before being challenged with 100 μM SIN-1 at 37°C for 30 min. The extent of oxidative damage to DNA was evaluated by comet assay, as described in Section 2. Data represent the mean ± S.E.M. of four experimental determinations. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

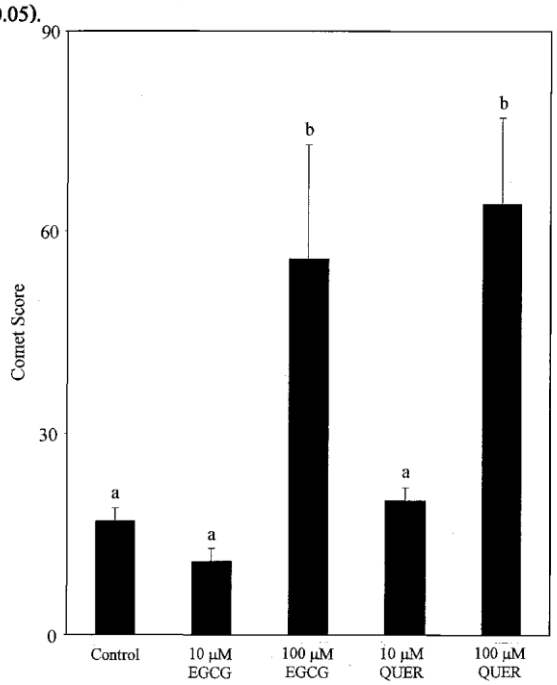

Fig. 6. Damaging effects of high concentrations of either EGCG or quercetin on DNA in Jurkat T-lymphocytes. Cells were incubated with 10 or 100 μM of either EGCG or quercetin at 37°C for 30 min. The extent of DNA damage was evaluated by comet assay, as described in Section 2. Data represent the mean ± S.E.M. of four experimental determinations. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

Finally, we determined if 10-fold higher concentrations (i.e., 100 μM) of EGCG or quercetin by themselves induce damage to DNA in Jurkat T-lymphocytes (Fig. 6). Compared to control cells (comet score: 17 ± 2), EGCG- and quercetin-treated cells had comet scores of 56 ± 17 and 64 ± 13, respectively. However, cells treated with low concentrations (i.e., 10 μM) of EGCG and quercetin had comet scores of only 15 ± 2 and 20 ± 2, respectively.

4. DISCUSSION

Phenolic phytochemicals are a large group of substances found in many fruits, vegetables, spices, and herbs. They are of research interest largely because of their antioxidant activity [13]. Numerous studies reviewed elsewhere [14,15] suggest that diets rich in antioxidants such as the phenolic phytochemicals reduce risk of chronic degenerative diseases often associated with free radicals. For example, it is possible that phenolic phytochemicals inhibit oxidative DNA damage, mutagenesis, and carcinogenesis [15]. Due to their diverse chemical structures, phenolic phytochemicals likely possess different degrees of antioxidant activity. Hesperetin, naringenin, genistein, daidzein, quercetin, and EGCG were studied because they have similar core structures, although distinct differences in constituent groups can also be seen (Fig. 1). Moreover, these phenolic phytochemicals are present in some popular foods worldwide, including citrus products, soybeans, onions, and green tea most notably.

Upon initially screening the phenolic phytochemicals for DPPH-scavenging capacity, EGCG and quercetin were effective scavengers, in contrast to hesperetin, naringenin, genistein, and daidzein. Any scavenging action reflects the ability of the phenolic phytochemicals to provide a hydrogen atom to the DPPH radical, which results in decoloration and decrease in absorbance of DPPH in solution [10]. As such, the results of this first experiment may be related to the chemical structures of the phenolic phytochemicals (Fig. 1). The general notion is that the DPPH radical-scavenging activity of the phenolic phytochemicals is primarily determined by the number of phenolic hydroxyl groups, which are the active centers of the molecules in terms of furnishing hydrogen atoms to react with the DPPH radical. Indeed, EGCG with eight phenolic hydroxyl groups was the most potent scavenger of the DPPH radical, while quercetin with five phenolic hydroxyl groups was the second most potent. Having only three or fewer phenolic hydroxyl groups, hesperetin, naringenin, genistein, and daidzein did not noticeably scavenge DPPH. Therefore, additional study was not performed on these four weaker phenolic phytochemicals. Instead, EGCG and quercetin were examined further.

To study the antioxidant effects of EGCG and quercetin in a cellular model, two different reagents (H_2O_2 and SIN-1) were used to induce oxidative damage to DNA in Jurkat T-lymphocytes that had been pre-treated with either one of the phenolic phytochemicals. In distinct reaction sequences, H_2O_2 and SIN-1 form different oxidizing species that ultimately damage DNA. H_2O_2 reacts with ferrous ion via the Fenton reaction to generate hydroxy radical [16]. As depicted figuratively [17], hydroxyl radical attacks the deoxyribose moiety of DNA to cause strand breaks. SIN-1 requires thermal decomposition to become active as an oxidant [8]. When incubated at 37°C, SIN-1 generates nitric oxide and superoxide anion. These two products react rapidly to form peroxynitrite that damages DNA. Apparently, peroxynitrite can also generate hydroxyl radical, but in a manner not requiring iron [18]. Therefore, H_2O_2 damages DNA via hydroxyl radical produced in the presence of metal ions. SIN-1 damages DNA via peroxynitrite and possibly hydroxyl radical, both produced from SIN-1 without involvement of metal ions.

In agreement with their abilities to effectively scavenge DPPH, low concentrations (i.e., 10 μM) of either EGCG or quercetin inhibited H_2O_2- and SIN-1-induced damage to DNA in Jurkat T-lymphocytes. The antioxidant effects of EGCG and quercetin are consistent with those of other phenolic phytochemicals, which have been reported to scavenge H_2O_2, [5], hydroxyl radical [8], superoxide anion [5], nitric oxide [3], and peroxynitrite [4]. Thus, in inhibiting the cellular DNA damage, EGCG and quercetin likely scavenged the intermediate or ultimate oxidizing species formed from H_2O_2 and SIN-1. Based on its better DPPH-scavenging capability, EGCG would have been expected to be superior to quercetin in inhibiting oxidative DNA damage in

the Jurkat T- lymphocytes, but this was not the case. Despite being weaker than EGCG in scavenging DPPH, quercetin was equally as potent as EGCG in inhibiting cellular DNA damage induced by either H_2O_2 or SIN-1.

On the contrary, it cannot be definitively concluded from the cellular experiments that quercetin was equally potent as EGCG on a mole-to-mole basis in inhibiting oxidative DNA damage. An important issue needs to be considered. Because the assays were not done, it is unknown if the actual concentrations of EGCG and quercetin were the same in the Jurkat T-lymphocytes after being pre-incubated separately with each substance at equimolar concentrations. From a structural perspective, it is conceivable that the cellular concentration of quercetin could have been higher than EGCG. Since it has a smaller number of phenolic hydroxyl groups, quercetin may be somewhat less polar than EGCG. Hence, the greater hydrophobicity of quercetin would allow it to better cross cell membranes. As such, the higher number of quercetin molecules in the cells would make quercetin appear just as potent as EGCG in inhibiting oxidative DNA damage. It is noteworthy that, in work with plasmid DNA [9], 50 μM EGCG and quercetin inhibited strand breaks by 64% and 42%, respectively, when the plasmid DNA damage was induced with peroxynitrite reagent. Thus, this previous non-cellular finding suggests that, relative to EGCG, quercetin may not be a better protector of DNA against oxidative damage if the cellular concentrations of the two phenolic phytochemicals are equivalent. On the other hand, assuming that quercetin and EGCG were taken up equally by the cells, EGCG may simply not be a better scavenger than quercetin of the reactive oxygen and nitrogen species tested in our cellular model containing a complex array of other bioactive molecules. A direct comparison between EGCG and quercetin in scavenging hydroxyl radical and peroxynitrite is unavailable.

The mechanism by which EGCG and quercetin inhibited oxidative damage to DNA in Jurkat T- lymphocytes can be surmised from their chemical structures. EGCG and quercetin may work by providing hydrogen atoms from their phenolic hydroxyl groups to scavenge hydroxyl radical generated from H_2O_2 and peroxynitrite generated from SIN-1. Many other phenolic phytochemicals work as antioxidants in this manner [13]. Another possible way that EGCG and quercetin inhibited oxidative damage to cellular DNA, particularly that induced by H_2O_2, may be by chelating metal ions which facilitate formation of hydroxyl radical from H_2O_2 [16]. The metal ion-chelating properties of phenolic phytochemicals have been demonstrated [19]. Analysis of the spectra absorbance of phenolic phytochemicals in the presence of metal ions revealed that quercetin, rutin, kaempferol, and catechin bound Cu(II) while quercetin and rutin bound Fe(II), Fe(III), and Mn(II).

Another part of the present study corroborated that the effects of certain phenolic phytochemicals can be contradictory. In non-cellular models, phenolic phytochemicals can have both antioxidant and pro-oxidant effects depending on the experimental conditions [8,9]. EGCG and quercetin had such effects in the experiments with Jurkat T-lymphocytes. Relatively low concentrations of 10 μM EGCG or quercetin each inhibited both H_2O_2- and SIN-1-induced DNA damage. Moreover, by themselves, 10 μM EGCG or quercetin did not induce DNA damage, in contrast to 100 μM EGCG or quercetin. Thus, it was not possible to totally inhibit H_2O_2- and SIN-1-induced DNA damage with concentrations of EGCG and quercetin higher than 10 μM because these phenolic phytochemicals assumed the role of pro-oxidants.

The pro-oxidant effects of relatively high concentrations of EGCG and quercetin seem to be due to the presence of metal ions and generation of reactive oxygen species. For instance, in the presence of iron or copper, quercetin induced DNA damage in isolated rat liver nuclei [20]. In a related study [9] indicating a requirement for metal ions in phenolic phytochemical-induced DNA damage, no strand breaks were detected in plasmid DNA incubated with 100 μM of either EGCG or quercetin but in the presence of diethylenetriamine pentaacetic acid that strongly chelates metal ions. The strand breaks were detected by noting the conversion of circular super-coiled DNA to a relaxed open circular form, observable on a UV-transilluminator after agarose gel electrophoresis and staining of the plasmid DNA with ethidium bromide. Additionally, it was shown that 50 μM of either EGCG or quercetin inhibited plasmid DNA strand breaks, when the DNA damage was induced by peroxynitrite reagent. These previous findings are now complemented by our cellular data, which show that both EGCG and quercetin inhibited DNA damage, but induced by SIN-1 and evaluated with the comet assay.

While this study was in progress, it has been reported in other experimental models that quercetin inhibits oxidative damage to cellular DNA as evaluated with the comet assay. Pre-treating HepG2 liver cells with 10 μM quercetin inhibited H_2O_2-induced DNA damage [21]. Similarly, pre-treating U-937 monocytic cells with 10 μM quercetin inhibited tert-butylhydroperoxide-induced DNA damage [22]. Quercetin also inhibited H_2O_2-induced DNA damage in human peripheral blood lymphocytes [23,24]. Therefore, our findings either confirm or extend these earlier studies.

In summary, of the phenolic phytochemicals tested, both EGCG and quercetin had substantial DPPH radical-scavenging or antioxidant activity. Using a cell culture system, low concentrations of EGCG and quercetin inhibited DNA damage induced by reactive oxygen and nitrogen species. In contrast, high concentrations of these two phenolic phytochemicals by themselves actually caused cellular DNA damage.

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