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Dietary Antioxidants Fail in Protection against Oxidative Genetic Damage in *In Vitro* Evaluation

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Carcinogenesis is believed to be induced through the oxidative damage of DNA, and antioxidants are expected to suppress it. So, the polyphenolic antioxidants in daily foods were investigated to see whether they protect against genetic damage by active oxygen. In the evaluation, we used a bioassay and a chemical determination, a *Salmonella* mutagenicity test for mutation by a *N*-hydroxyl radical from one of the dietary carcinogens 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole and the formation of 8-hydroxyl (8-OHdG) from 2′-deoxyguanosine (2′-dG) in a Fenton OH-radical generating system. Thirty-one antioxidants including flavonoids were compared in terms of radical-trapping activity with bacterial DNA and 2′-dG. Antioxidants inhibited the mutation but the IC_{50} values were in the mM order. Against 8-OHdG formation, only α-tocopherol had a suppressive effect with an IC_{50} of 1.5 μM. Thus, except α-tocopherol, the dietary antioxidants did not scavenge the biological radicals faster than bacterial DNA and intact 2′-dG, indicating that they failed to prevent oxidative gene damage and probably carcinogenesis.

Key words: flavonoid; 8-OHdG; hydrogen peroxide; antioxidant; genotoxicity

Carcinogenesis has been found to be induced through the oxidative damage of DNA, and antioxidants scavenge active oxygens.[1-5] It is expected therefore that dietary antioxidants can prevent cancer. However, the intake of polyphenolic antioxidants such as flavonoids was protective against cancer in only one prospective study.[6-8] In fact, it was inversely associated with coronary heart disease in most. This disease is also attributed to the active oxygens that cause peroxidation in low-density lipoprotein accompanying atherosclerosis. Other studies have provided evidence that antioxidants protect against atherosclerosis by inhibiting peroxidation.[9,10] Thus, in coronary heart disease dietary antioxidants are available in the body to scavenge active oxygens, whereas in carcinogenesis they are not.

There may be a misunderstanding as to the scavenging potency of the antioxidants. In terms of anti-carcinogenic action, antioxidants should be evaluated as to anti-genotoxic potency, that is, whether the antioxidant can scavenge active oxygens more easily than DNA.

In this study, we used two methods for evaluating anti-genotoxic potency; a bioassay with a *Salmonella* mutagenicity test and a chemical measurement of 8-hydroxy guanosine (8-OHdG) formation. For the former method, we used a *N*-hydroxyl form of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (N-OH-Trp-P-2) as an active oxygen. This is an ultimately carcinogenic form derived from a dietary carcinogen, heterocyclic amines, that have been implicated in human carcinogenesis.[11-13] Heterocyclic amines are activated by cytochrome P450 (CYP) monooxygenases to *N*-hydroxyl forms and have genotoxicity through the generation of radicals from the *N*-hydroxyls.[14,15] Antioxidants were evaluated for their inhibitory effect on *Salmonella* mutation induced by N-OH-Trp-P-2. Regarding the latter method, many studies have used 8-OHdG as a biomarker for oxidative gene damage including cancer.[16-18] We then used 2′-deoxyguanosine (2′-dG) as a target and generated OH radicals in a Fenton reaction, and evaluated the suppressing activity of antioxidants on 8-OHdG formation.

Thirty-one polyphenols were tested by comparing the damaging sensitivities of the bacterial DNA and 2′-dG. They occur in the daily diet and have been recognized as strong antioxidants; catechin in teas,[19] caffeic and chlorogenic acids in coffee,[20] curcumin in the herb turmeric,[21] dopamine in banana,[22] flavones and flavonols in vegetables and fruits,[23] sesamol in sesame,[24] and so forth. Unexpectedly, almost all of them failed as anti-genotoxicants.

Materials and Methods

Materials. Antioxidants were purchased as fol-

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lows: anthracene, anthrone, anthraquinone, ascorbic acid, butylated hydroxyanisole (BHA), (+)-catechin, chlorogenic acid, danthron, and dopamine were from Nacalai Tesque. (Kyoto, Japan). Apigenin and nordihydroguaiaretic acid (NDGA) were obtained from Sigma (St. Louis, MO). Butylated hydroxytoluene (BHT), caffeic acid, β-carotene, curcumin, cysteine, kaempferol, quercetin, rutin, and DL-α-tocopherol were from Wako Pure Chemical Ind. (Tokyo, Japan). Chrysophanol, flavonol, emodin, galangin, luteolin, myricetin, naringenin, and rhein were from Funakoshi (Tokyo, Japan). n-Butyl gallate and *tert*-butylhydroquinone (TBHQ) were from Tokyo Kasei Kogyo (Tokyo, Japan). Sesamol was from Aldrich (Tokyo, Japan). The target for the OH radical 2′-dG was obtained from Sigma. Its product 8-OHdG was obtained from Wako Pure Chemical as a standard chemical. The stable radical reagent 1,1-diphenyl-2-picrylhydrazyl (DPPH) was from Nacalai Tesque. All other chemicals were of the highest grade commercially available.

Antioxidative potency measured with DPPH. First we measured the antioxidative potency with a classic method using DPPH radicals.[25] Six different amounts of antioxidants in ethanol were added to 250 nmol DPPH in 50% ethanol in 0.05 M acetate buffer (pH 5.5), and immediately the fading at 517 nm was monitored.

Scavenging activity toward N-OH-Trp-P-2. The scavenging activity of antioxidants toward N-OH-Trp-P-2 radicals before the attack on the DNA was evaluated with *Salmonella typhimurium* strain TA98. The N-OH-Trp-P-2 was prepared by an incubation of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) with microsomes obtained from the recombinant yeast *Saccharomyces cervisiae* strain AH22/pA1A1IRR that expressed human CYP1A1 and yeast NADPH-CYP-oxidoreductase simultaneously as described previously.[26] The yeast microsomes (0.1 mg as protein) produced 3.7 pmol of N-OH-Trp-P-2 in a 5-min incubation with 25 pmol Trp-P-2 at 37°C, when measured by our previous method using a HPLC and electrochemical detector.[27] After the incubation, the microsomal solution was placed in boiling water for 20 sec to inactivate the enzymes, cooled to room temperature, and then added to the TA98 bacterial suspension (0.1 ml) together with antioxidants. After incubation for another 20 min, the suspension was mixed with 2 ml of molten top agar and poured onto an agar medium of minimal glucose. The His^+-revertant colonies were counted after a 2-day culture. The scavenging activity of antioxidants toward N-OH-Trp-P-2 radicals was evaluated from the suppression of *Salmonella* mutation as follows: $[(A-B)-(C-D)]/(A-B)\times 100$, where A is the number of revertants obtained with N-OH-Trp-P-2 (1763 ± 132, mean ± SD), B is the number of spontaneous revertants, C is the number of revertants obtained with both antioxidants and N-OH-Trp-P-2, and D is the number of revertants obtained with antioxidants. When the six different concentrations of antioxidants were used to plot a dose-response curve, the IC_{50} values were calculated by plotting the suppressing activity against the log of the dose amount as mentioned previously.[28] The IC_{50} values are the amounts of antioxidants required for 50% scavenging of N-OH-Trp-P-2 radicals before damage to the bacterial DNA. The assays were done independently in triplicate.

Effects on 8-OHdG formation of OH radicals. Another evaluation method for anti-genotoxicity used 2′-dG as a target and the Fenton reaction for OH radical generation, modifying the method of Kasai and Nishimura[29] with respect to reagent concentrations. A reaction mixture containing 0.25 mM 2′-dG, 0.05 mM H_2O_2, 0.13 mM $FeSO_4$, and 0.65 mM ethylenediaminetetraacetate disodium salt in 1 ml of 10 mM soidum phosphate buffer (pH 6.8) was incubated at 37°C for 15 min. Passing through a membrane filter (pore size: 0.2 μm, 13A, GL Science Inc., Tokyo Japan), a 20-μl sample was analyzed on a HPLC: column, Inertsil (4.6×250 mm) maintained at 45°C; solvent system, sodium phosphate buffer (pH 5.1) in 10% methanol; and flow rate, 1.0 ml/min. An electrochemical detector set at +550 mV and uv monitor at 254 nm were used simultaneously. The former specifically measured 8-OHdG (up to 1 pmol) at 13.54 min of retention time and the latter 2′-dG at 9.57 min. In the system, various concentrations of the antioxidants in dimethylsulfoxide (vehicle amount was finally 1 μl) were added, and compared in the formation of 8-OHdG to vehicle alone that produced 430 nmol 8-OHdG in the 15-min incubation.

Results

Scavenging ability of antioxidants for DPPH radicals

Among 31 dietary antioxidants, we compared the antioxidative potency quantitatively by a classic method using DPPH as shown in Fig. 1. Some antioxidants such as quercetin scavenged DPPH radicals slowly (Fig. 1A) while others such as ascorbic acid did so rapidly (Fig. 1B), but both had a dose-dependent effect. For a quantitative comparison, the scavenged amounts of DPPH were measured within 1-min of incubation and were plotted against the dosages of antioxidants. The amount required for 50% scavenging, the IC_{50}, was calculated as shown in Fig. 1C. Table 1 summarizes the IC_{50} values for the 31 antioxidants alphabetically comparing to 2′-dG. 2′-dG had no activity for the DPPH radical. Com-

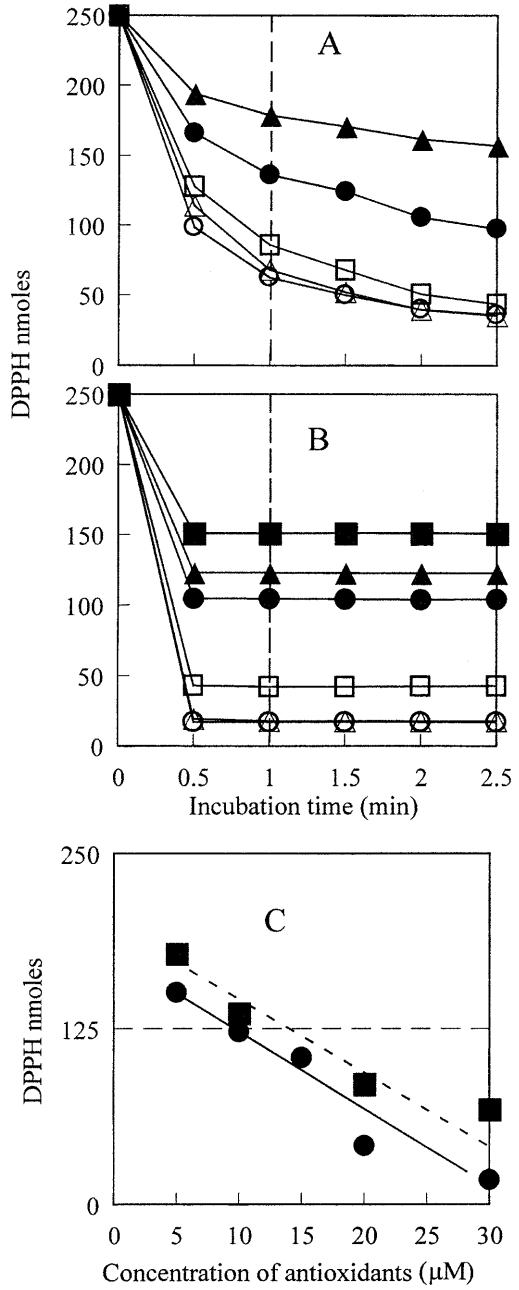

Fig. 1. Measurement of IC_{50} Values for Antioxidants to Scavenge DPPH Radical.

The antioxidants were added to an incubation mixture containing 250 nmoles DPPH, and the reduction of DPPH radical immediately measured photometrically as mentioned in "Materials and Methods". A: A typical result with quercetin at the concentrations of ○, 50; △, 30; □, 20; ●, 10; and ▲, 5 μM. B: A typical result with ascorbic acid at ○, 50; △, 30; □, 20; ●, 15; ▲, 10; and ■, 5 μM. C: The reduction in DPPH radical within 1 min was plotted against the respective concentrations of antioxidants. The IC_{50} values were then measured as the concentration of antioxidants required for scavenging 50% of DPPH radicals in the solution (125 nmoles) within 1 min, being 11.0 μM for ascorbic acid and 14.0 μM for quercetin.

pounds having a catechol structure are well known to be strong antioxidants.[22,30] In this experiment, the catechols such as caffeic acid, dopamine, myricetin, gallic acid, and NDGA had stronger activity and scavenged DPPH radicals more easily than 2′-dG. These compounds may protect DNA from oxidative damage. However, DPPH is an artificial radical which does not occur in biological systems. An important issue is whether the antioxidants can scavenge biological radicals more rapidly than 2′-dG.

Table 1. Scavenging IC_{50} Values for Dietary Antioxidants to DPPH Radical[a]

	IC_{50} (μM)		IC_{50} (μM)
2′-dG	no effect[b]	Cysteine	110
Ascorbic acid	11	Dopamine	6.8
Anthraquinones		Flavones	
anthracene	no effect[b]	apigenin	no effect[b]
anthrone	230	luteolin	22
anthraquinone	no effect[b]	Flavonols	
chrysophanol	no effect[b]	flavonol	no effect[b]
danthron	no effect[b]	galangin	110
emodin	no effect[b]	kaempferol	17
rhein	no effect[b]	myricetin	8.0
BHA	46	quercetin	14
BHT	69	rutin	18
TBHQ	22	Flavanones	
Caffeic acid	8.0	naringenin	no effect[b]
β-Carotene	no effect[b]	Gallic acid n-butyl ester	6.0
(+)-Catechin	16	NDGA	6.5
Chlorogenic acid	22	Sesamol	25
Curcumin	37	α-Tocopherol	11

[a] For the determination of IC_{50} values, see Fig. 1.
[b] "no effect" up to 500 μM.
Abbreviations for food additives: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, *tert*-butylhydroquinone; NDGA, nordihydroguaiaretic acid.

Effects on the N-OH-Trp-P-2 radical

Heterocyclic amines such as Trp-P-2 are the most abundant dietary procarcinogens.[31] Humans are estimated to take in 0.4–16 μg of heterocyclic amines per day per capita.[12] They are activated metabolically to carcinogenic forms such as N-OH-Trp-P-2. N-OH-Trp-P-2 easily generates radicals and attacks DNA, subsequently inducing a frame-shift mutation.[14,15]

Then, we used N-OH-Trp-P-2 as a biological radical, and detected the DNA damage with a bioassay using the *Salmonella* mutagenicity test. Table 2 shows the scavenging activity of antioxidants against N-OH-Trp-P-2 before attacking the bacterial DNA. 2′-dG had no effect and most antioxidants tested here scavenged N-OH-Trp-P-2 dose-dependently. However, the IC_{50} values were large and in the order of mM. Values in mM were given even by ascorbic acid and β-carotene, and ineffective for α-tocopherol, though they were recognized generally as being the most significant biological-antioxidants.[32] Only emodin, myricetin, and quercetin gave IC_{50} values from 30–80 μM.

Table 2. Inhibitory IC_{50} Values of Dietary Antioxidants toward *Salmonella* Mutation Induced by N-OH-Trp-P-2 Radical

	IC_{50} (mM)		IC_{50} (mM)
2′-dG	no effect[a]	Cysteine	no effect[a]
Ascorbic acid	3.4	Dopamine	no effect[a]
Anthraquinones		Flavones	
anthracene	3.7	apigenin	no effect[a]
anthrone	2.2	luteolin	2.4
anthraquinone	9.2	Flavonols	
chrysophanol	2.4	flavonol	7.4
danthron	0.80	galangin	34
emodin	0.08	kaempferol	4.9
rhein	0.20	myricetin	0.03
BHA	1.6	quercetin	0.06
BHT	6.1	rutin	no effect[a]
TBHQ	0.70	Flavanones	
Caffeic acid	13	naringenin	2.1
β-Carotene	0.14	Gallic acid n-butyl ester	1.6
(+)-Catechin	5.4	NDGA	0.35
Chlorogenic acid	no effect[a]	Sesamol	0.20
Curcumin	0.40	α-Tocopherol	no effect[a]

[a] "no effect" up to 50 mM.

Abbreviations for food additives: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, tert-butylhydroquinone; NDGA, nordihydroguaiaretic acid.

Prevention of 8-OHdG formation by OH radical

As another biological radical we used the OH radical in the Fenton reaction system. Figure 2 shows the effects of antioxidants on the formation of 8-OHdG from 2′-dG. In the absence of antioxidants, 2′-dG formed 0.43 nmol of 8-OHdG in the 15-min incubation. Among the antioxidants tested here, only α-tocopherol suppressed the formation dose-dependently (Fig. 2A), and gave 1.5 μM for the IC_{50} value. Luteolin and kaempferol suppressed the formation by 51% and 24%, respectively, at the concentration of 10 μM (Fig. 2A). Quercetin, rutin, and naringenin suppressed it by 30, 27, and 33%, respectively (Fig. 2B). They, however, did not show more suppressing activity at higher concentrations, 100 μM or more (data not shown), and thus did not give IC_{50} values. Some compounds increased the formation; catechin and gallic acid in Fig. 2D, caffeic acid and chlorogenic acid in Fig. 2E, and ascorbic acid and cysteine in Fig. 2F. Ascorbic acid facilitated the action of OH radicals and increased the formation dose-dependently between concentrations of 0.03 and 3 mM (Fig. 3). The other compounds in Fig. 2 and seven anthraquinones were almost ineffective. We have further experimented under other conditions, more drastic or mild, such as the formation of 4.5 or 0.18 nmol of 8-OHdG in 15-min incubation, and found that the antioxidants did not suppress the formation (data not shown). Thus, except α-tocopherol, all of the dietary antioxidants tested here failed to scavenge OH radicals before 2′-dG was damaged, and several of them increased the damage.

Antioxidants at various concentrations should coexist in the body with biological components such as ascorbic acid and cysteine. So, we set systems containing 0.1 mM ascorbic acid (Fig. 4A), 2.0 mM cysteine (Fig. 4B) and both (Fig. 4C), and added five antioxidants. Luteolin, quercetin, catechin, chlorogenic acid, and sesamol did not change the rate of 8-OHdG formation in Fig. 4A. Similarly, they kept the formation near the level of 0.43 nmol (Fig. 4B). In the system containing both ascorbic acid and cysteine (Fig. 4C), they increased the 8-OHdG formation at higher concentrations but only a little. Luteolin and quercetin themselves alone had been antioxidative, and catechin and chlorogenic acid prooxidative (Fig. 2). On combining the systems with ascorbic acid and/or cysteine, as in Fig. 4, however, they changed character. These results indicated that the antioxidants themselves facilitated the oxidative action of the OH radical or somewhat inhibited its action, and counteracted both the actions when with the biological components ascorbic acid and cysteine. Subsequently, the dietary antioxidants seem unlikely to either increase or protect against the genetic damage by OH radicals in the body.

Discussion

This study reveals that dietary antioxidants such as flavonoids do not scavenge active oxygens faster than bacterial DNA and 2′-dG, while only a classic antioxidant, α-tocopherol, effectively scavenged OH radicals (IC_{50} of 1.5 μM). This means that the newly recognized antioxidants fail to protect against oxidative gene damage, and well explains the analytical data in epidemiological studies in which intake of polyphenolic antioxidants such as flavonoids was not protective against cancer.[6-8]

Against the DPPH radical, caffeic acid, catechin, dopamine, several flavonols, and gallic acid were as effective as the classic antioxidants, ascorbic acid, β-carotene and α-tocopherol (Table 1). However, it is doubtful whether the results reflect biologically available activity, because the DPPH radical does not occur in any biological systems. Then, we used biologically occurring radicals, N-OH-Trp-P-2 and OH. The antioxidants inhibited the bacterial mutations induced by the N-OH-Trp-P-2 radical but the effective concentrations were mostly in the mM order (Table 2). Only quercetin and myricetin showed 30 and 60 μM as IC_{50} values for trapping the N-OH-Trp-P-2 radical, respectively. These concentrations, however, may not be physiologically available. Hollman and coworkers[33,34] have found in absorption experiments in volunteers that fried onion containing the equivalent of 68 mg (225 μmol) of quercetin gives 0.74 μM in plasma as a peak at 0.7–7 h after the ingestion. Conquer *et al.*[35] have reported that a capsule containing free quercetin raised the concentration to 1.5 μM after 28 days even on repeated dos-

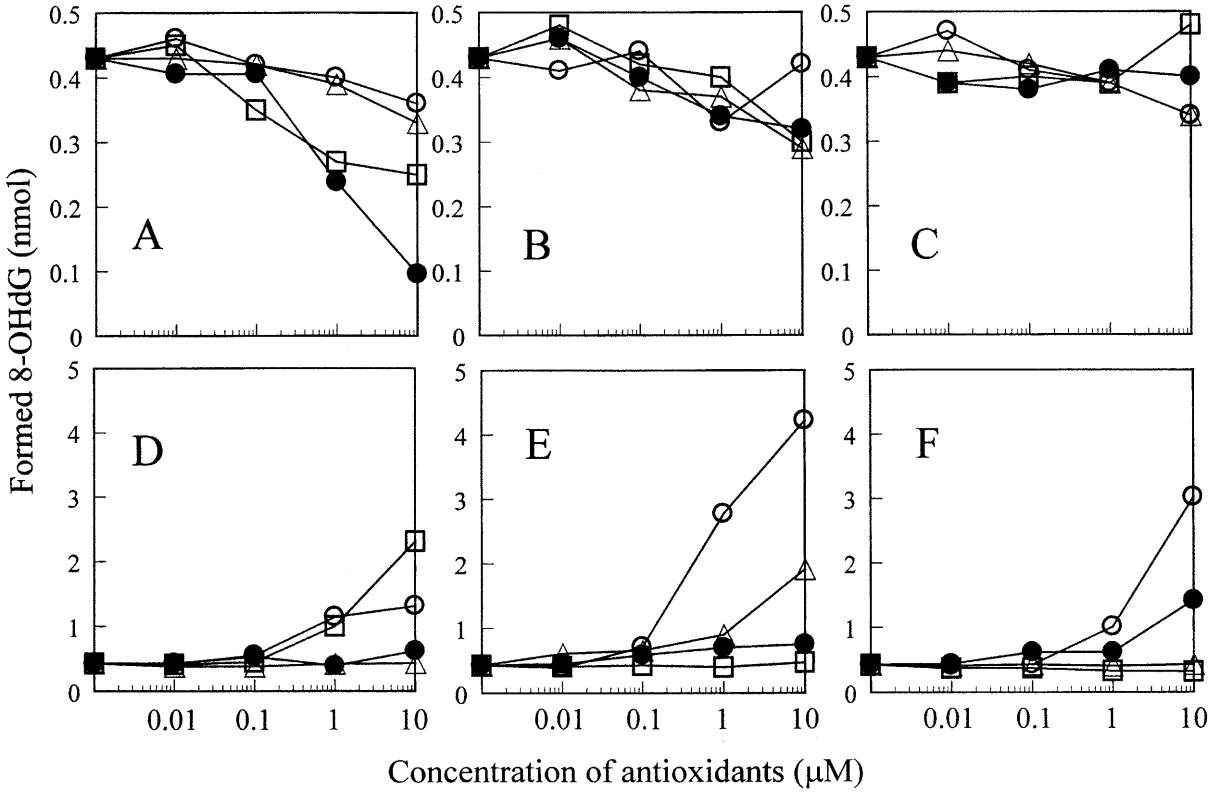

Fig. 2. Effects of Dietary Antioxidants on 8-OHdG Formation by OH Radicals Generated through the Fenton Reaction.

In the Fenton reaction system containing 0.25 mM 2′-dG, antioxidants in 1 μl of dimethylsulfoxide were incubated at 37°C for 15 min, and the amount of 8-OHdG formed was measured as mentioned in Materials and Methods. The incubation with vehicle alone produced 0.43 nmoles 8-OHdG. Among antioxidants tested here, only α-tocopherol suppressed the formation dose-dependently and gave an IC_{50} value of 1.5 μM. A: the effects of ○, apigenin; △, kaempferol; □, luteolin; and ●, α-tocopherol. B: ○, galangin; △, naringenin; □, quercetin; and ●, rutin. C: ○, butylated hydroxyanisole; △, butylated hydroxytoluene; □, *tert*-butylhydroquinone; and ●, nordihydroguaiaretic acid. D: ○, catechin; △, flavonol; □, gallic acid n-butyl ester; and ●, myricetin. E: ○, caffeic acid; △, chlorogenic acid; □, dopamine; and ●, sesamol. F: ○, ascorbic acid; △, β-carotene; □, curcumin; and ●, cysteine.

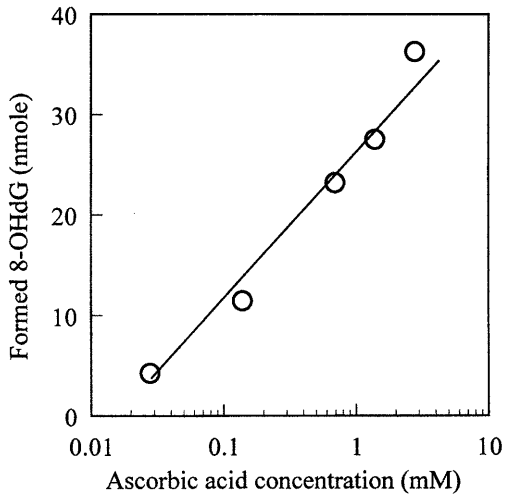

Fig. 3. Action of Ascorbic Acid in Increasing 8-OHdG Formation in the Fenton System.

In the same system as in Fig. 2, various concentrations of ascorbic acid were incubated with 2′-dG at 37°C for 15 min.

ing with 1 g per day. This shows that physiological levels of flavonoids such as quercetin are difficult to attain at 30 μM or more in humans. Thus, dietary polyphenols probably fail to inhibit genetic damage by N-OH-Trp-P-2, and subsequently, to prevent dietary carcinogenesis.

As another biological radical, generating it in the Fenton system used the OH radical. In human body, ferrous ion is usually a chelated complex with proteins such as ferritin. We here used ethylenediaminetetraacetate disodium salt in the Fenton system at the 5-fold excess amount by molar concentration against ferrous ion. This experimental system probably reflects the biological conditions. Against the OH radical, only α-tocopherol exhibited appreciative levels of activity: the others were almost ineffective (Fig. 2). Kaempferol, quercetin, rutin, and naringenin appeared to be effective at 10 μM, and luteolin scavenged the radical at 1 μM (Fig. 2A). The 10 μM concentration is also difficult to achieve in humans. Dietary luteolin may protect against genetic damage by OH radicals. α-Tocopherol appears to be available in humans, and Mascio *et al.* have reported that α-tocopherol is an effective scavenger for peroxyl radicals.[32]

Ascorbic acid and cysteine are biological components and present at intracellular concentrations of

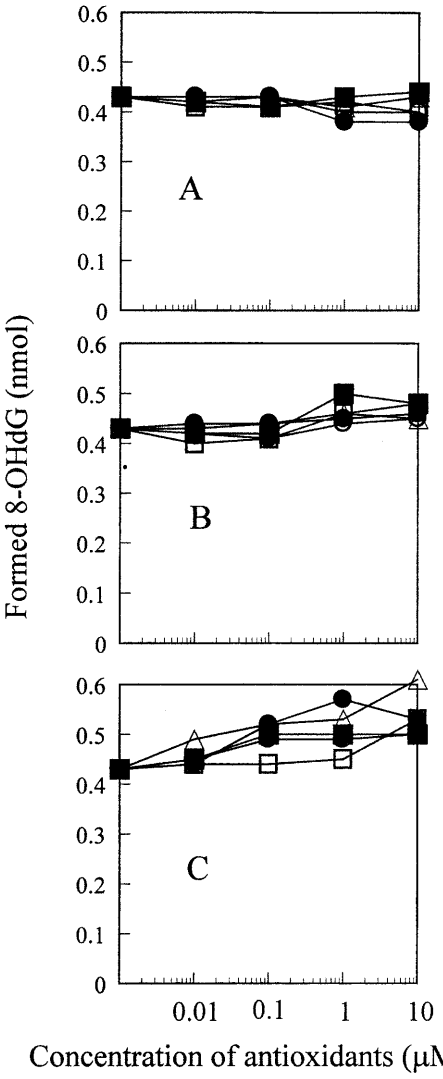

Fig. 4. Anti-genotoxic Effects of Antioxidants in a System Combined with Ascorbic Acid and/or Cysteine.

In the same system as in Fig. 2, 0.1 mM ascorbic acid (A), 2.0 mM cysteine (B), or both (C) were present, and ○, luteolin; △, quercetin; □, catechin; ●, chlorogenic acid; and ■, sesamol were added and incubated at 37°C for 15 min, measuring the amount of 8-OHdG formed.

<3.5 mM and 0.5–10 mM (as glutathione), respectively.[36,37] Dietary antioxidants coexist with ascorbic acid and cysteine in the body. Under these conditions, the antioxidants lost their original actions, to suppress or increase the genetic damage by the OH radical (Fig. 4). Thus, the flavonoids tested here are not considered to have antioxidative activity in oxidative gene damage in the body.

Dietary antioxidants such as flavonoids had been well understood to be antioxidative on lipid peroxidation in low-density lipoproteins.[9,10] It is a fact that they prevent cardiovascular diseases through antioxidative action.[6,7] However, they have been reported not to prevent human cancer.[6,7] As this study shows that they can not scavenge N-OH-Trp-P-2 and OH radicals before damaging DNA and 2′-dG, it is considered that the genes are more sensitive to biological radicals than coexistent antioxidants.

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