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A Novel Method Using 8-hydroperoxy-2′-deoxyguanosine Formation for Evaluating Antioxidative Potency

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Degenerative diseases such as cancer are induced by oxidative genetic damage. Antioxidants can scavenge reactive oxygen species, but to prevent disease, they must do so quickly, before the DNA bases are damaged. In the present study, a novel method was established for evaluating the potency of antioxidants employing 2′-deoxyguanosine as a target and 2,2′-azobis(2-amidinopropane) dihydrochloride as a reactive oxygen generator. The reaction formed one product linearly with time. This product was a novel 8-hydroperoxy-2′-deoxyguanosine (8-OOHdG). Using this system, 81 antioxidants occurring in our diet were assayed for activity to suppress the formation of 8-OOHdG by high-performance liquid chromatography (HPLC). The system was useful for the evaluation of antioxidative potency, compared to another method utilizing 1,1-diphenyl-2-picrylhydrazyl (DPPH). Further, it was enabled to examine the synergism of antioxidants. The formation of 8-OOHdG started only after the antioxidants had been consumed. Ascorbic acid, quercetin, and epigallocatechin gallate together delayed the formation by the sum total of the delay times of each factor alone. The proposed method is simple and easy, and can evaluate which dietary antioxidants inhibit reactive oxygen species more quickly than the DNA bases are damaged.

Keywords: Antioxidant; Oxidative stress; 8-Hydroperoxy-dG; Genetic damage; Flavonoids; Polyphenols

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; 2′-dG, 2′-deoxyguanosine; DMSO, dimethylsulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EGCG, (−)-epigallocatechin gallate; HPLC, high-performance liquid chromatography; 8-OHdG, 8-oxo-7,8-dihydrodeoxyguanosine (8-hydroxy-2′-deoxyguanosine); 8-OOHdG, 8-hydroperoxy-2′-deoxyguanosine

INTRODUCTION

Cancer and other degenerative diseases are believed to be induced by oxidative genetic damage. The oxidative damage is caused by reactive oxygen species,[1–3] and is mainly due to the formation of 8-hydroxyls, 8-oxo-7,8-dihydrodeoxyguanosine (8-hydroxy-2′-deoxyguanosine, 8-OHdG) and/or 8-hydroxy-2′-deoxyadenosine, in the bases.[4–7] An accumulation of 8-OHdG has been found in several carcinoma cells and mutated genes.[8–10] Levels of 8-OHdG in human skeletal muscle, respiratory epithelium, and leukocytes are correlated with aging, exposure to urban pollution, and smoking, respectively.[11–13] Urinary 8-OHdG is a biomarker of human oxidative stress accompanying cancer.[14–17]

Antioxidants can scavenge reactive oxygen species. The antioxidants in our diet may therefore prevent disease.[18] A better understanding is needed of which dietary antioxidants can trap reactive oxygen species effectively enough to prevent genetic damage, i.e. easier than DNA. The putative reactive oxygen species that form 8-hydroxy derivatives are hydrogen peroxide,[19–22] thiol radical,[23] and lipid peroxides.[24] However, they all require transition metals, UV rays, or γ rays to interconvert and generate oxidizing species, and several of them have to form hydrogen peroxide or lipid peroxides. Thus, Fenton's reaction has been employed using 2′-deoxyguanosine (2′-dG) as a target,[25] when evaluating antioxidants. This system is composed

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of several subfactors such as hydrogen peroxide, iron(II), and ethylenediaminetetraacetate disodium salt.[26,27] The various subfactors complicates the assay system and may result in errors during the evaluation, for example, several antioxidants interact with metal ions[28] and chelator.[29]

A simple system and convenient method are better. In the present study, we employed 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) because it has been proved to produce stoichiometrically a molecular oxygen radical via an intermediary AAPH-peroxyl radical.[30] The oxygen radical is present in normal cells at high steady-state concentrations.[31] Especially in mitochondria, side reactions of the electron transport chain with molecular oxygen directly generate its radical, superoxide anion.[32,33] Then, being 2′-dG as a target, it formed a novel product, 8-hydroperoxy-2′deoxyguanosine (8-OOHdG), quantitatively. The system could easily and accurately evaluate the potency of both water- and lipid-soluble antioxidants to suppress the formation by high-performance liquid chromatography (HPLC) analysis.

MATERIALS AND METHODS

Chemicals

AAPH, 2′-dG, 8-OHdG, and ascorbic acid were obtained from Wako Pure Chemical Ind. (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was from Nacalai Tesque (Kyoto, Japan). Quercetin was from Extrasynthèse (Genay, France), epigallocatechin gallate (EGCG) was from Kurita Kogyo (Tokyo, Japan), and the other antioxidants were high-grade commercial products. These products were tested to be free from impurities by HPLC before use. Water was distilled twice, and all other reagents used were of the highest grade available.

HPLC Analysis for Reaction Products of 2′-dG and AAPH

AAPH (25 mM) was mixed with 2′-dG (0.5 mM) in water and incubated at 37°C with stirring under atmospheric pressure. The pH of the mixture was 7.0 and remained unchanged during the incubation. An aliquot (10 μl) was analyzed in a reverse-phased HPLC system equipped with UV (254 nm) (Hitachi L-7100 and L-7420) and electrochemical (+600 mV) (IRICA Σ875; Kyoto, Japan) detectors. The conditions for HPLC were as follows: column, Capcell pak C18 UG120 (5 μm mesh and ϕ 4.6 × 250 mm) (Shiseido, Tokyo, Japan), maintained at 35°C; mobile phase, a mixed solvent of 6.5% methanol and 93.5% 20 mM potassium phosphate buffer (pH 4.5) containing 0.1 mM ethylenediaminetetraacetate disodium salt; and flow rate, 1.0 ml min^{-1}.

Purification of the Products

To obtain the reaction products, another mixture of 250 mM AAPH and 2.5 mM 2′-dG was incubated for 6 h. This mixture (10 ml) was passed through an ion-exchange column (Dowex 1 × 4, 50–100 mesh, OH form, ϕ 14 × 100 mm). The products were eluted with 0.1N HCl after a wash with 50 ml of 0.1N NaOH and 50 ml of water. The elutant was neutralized with 1N NaOH and concentrated by an evaporator to around 5 ml. It was then subjected to HPLC with a Cosmosil Packed Column (preparative column, ϕ 10 × 250 mm) (Nacalai Tesque, Kyoto, Japan) and eluted with water. The fraction showing absorption at 254 nm was collected and dried with a centrifugal concentrator (VC-96N, Taitec). The dried products showed a single peak in the above analytical HPLC which coincided with one of the product peaks.

Elucidation of Chemical Structure

The chemical structures of the dried products were elucidated using nuclear magnetic resonance (NMR) spectrometry (DPX-250, Bruker Analytik GmbH). Proton spectra were recorded at 250 MHz and ^{13}C at 62.5 MHz in D_2O and 3-trimethylsilylpropionate-2,2,3,3, D_4. Mass spectra were obtained with an HPLC–mass spectrometer (LC/MS M-1200H, Hitachi) under atmospheric pressure, with chemical ionization and ionizing at 30 eV. Infrared spectra were determined with a FT-IR (FTIR-8600 PC, Shimadzu) in KBr powder by a diffusion reflectance method.

Antioxidant Assay With 2′-dG and AAPH

Antioxidants in 5 μl of dimethylsulfoxide (DMSO) were added at six different concentrations to the mixture of 0.5 mM 2′-dG and 25 mM AAPH. After 1 h incubation at 37°C, the amount of 8-OOHdG formed was determined with 10 μl of the mixture using the analytical HPLC system at UV 254 nm. When the chemicals constructed a dose-response curve, the IC_{50} values were calculated by plotting the suppression of 8-OOHdG formation against the dose. The IC_{50} is the amount of antioxidant required for 50% suppression of 8-OOHdG formation. The assays were done independently in triplicate.

Another Assay With DPPH

The assay accuracy was compared to a convenient and widely used method employing the DPPH radical.[34] Six different amounts of each antioxidant in ethanol were added to 250 nmol DPPH in 50%

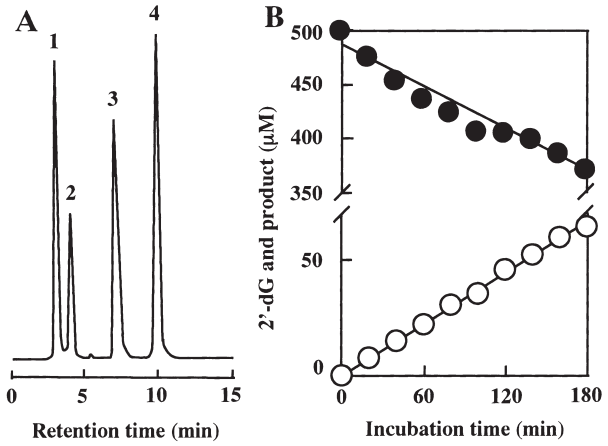


FIGURE 1 HPLC for reaction products of AAPH and 2′-dG. (A) A profile of 1 h incubation of 25 mM AAPH with 0.5 mM 2′-dG at 37°C. The incubation mixture (10 μl) was analyzed by reverse-phased HPLC with monitoring at 254 nm. Peaks 1–4 were identified as AAPH, a reaction product, decomposed products of AAPH, and 2′-dG, respectively. (B) Incubation time-dependent formation of the product (○) and decrease of 2′-dG (●).

ethanol in 50 mM acetate buffer (pH 5.5), and the signal was monitored at 517 nm. The reduction in DPPH radical within 5 min was plotted against the respective concentrations of antioxidant. The IC_{50} value was then calculated as the concentration of antioxidant required for scavenging 50% of DPPH radicals in the solution (125 nmol) within 5 min.

RESULTS

Reaction of AAPH and 2′-dG

Figure 1A is the HPLC profile obtained when the reaction mixture of AAPH and 2′-dG was incubated for 1 h and monitored at 254 nm. Other monitoring methods using different wavelengths and electrochemical detection which has been known to be sensitive to 8-OHdG,[35] did not give additional peaks. Thus, the reaction of AAPH and 2′-dG gave four detectable products on HPLC. The first peak at a retention time of 3.3 min was the original AAPH while the third at 7.8 min was its decomposed product, because the incubation of AAPH alone produced the same profile. The fourth at 10.1 min coincided with 2′-dG in retention time. A minor peak at 5.5 min was identified as guanine contaminating the reagent. Thus, the second peak at 4.3 min was the reaction product of AAPH and 2′-dG. The production involved time-dependent consumption of 2′-dG (Fig. 1B).

Identification of the Product

The characteristics of the purified product are summarized in Table I along with those of standard 2′-dG and 8-OHdG. The product gave a parent ion peak at m/z 300 suggesting an addition of 32 mass units (O_2) to 2′-dG. The UV spectrum indicated a purine skeleton as well as 2′-dG. Based on IR analysis, the bands were assigned to ν (O–O) and δ (OOH). Chemical determination of the peroxide value indicated one peroxide group per mole of the product. The value was negative in 2′-dG and 8-OHdG.

In NMR (Table II), the product gave no signal for H-8 or 8-OHdG. The other signals were assigned to ribose protons from H-1′ to H-5′,[37] which shifted slightly to higher magnetic fields compared to those of 2′-dG, with a larger shift for H-1′ than 8-OHdG. The shifts are a feature of substitution at the 8-position.[38,39] In carbon spectra, C-8 shifted greatly to a lower magnetic field compared to the original 2′-dG, and C-2, C-4, and C-5 to higher fields as well as 8-OHdG.[40] The shift of C-8 was greater than that in 8-OHdG. The proton and carbon spectra thus indicated a group other than OH substituted at the 8-position. The substitute was suggested to be a peroxide based on the data from mass and IR spectra and chemical determination.

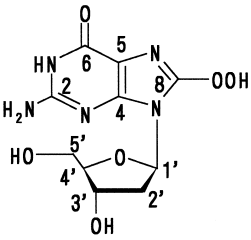
A question remaining is whether the substitute is a hydroperoxide or endoperoxide such as 4,8 or 5,8. Then, the product was incubated in 0.17N HCl at 37°C (Fig. 2). A 15-min incubation produced an appreciable amount of 8-OH-guanine and a small amount of 8-OHdG, and simultaneously the formation of 2′-deoxyribose was detected on thin layer chromatography with Merck Kieselgel 60 F_{254} (Darmstadt, Germany) developed in methanol:ethyl

TABLE I Spectrometric and chemical characters of the reaction product of AAPH and 2′-dG

Analysis	Product	2′-dG	8-OHdG
LC/MS (m/z)	$(M+1)^+$, 300 (M + 1)-ribose, 184	$(M+1)^+$, 268 (M + 1)-ribose, 152	$(M+1)^+$, 284 (M + 1)-ribose, 168
UV (H_2O): λ_{max} (ε)*	254 (15,300) and 313 (1670)	253 (12,800)	245 (12,300) and 293 (10,300)
ν (O–O)	900 cm^{-1}	No signals	No signals
δ (OOH)	1125 cm^{-1}		
Peroxide value (mol mol^{-1})†	0.93	Negative	Negative

*Molecular extinction coefficient of the product was calculated with the molecular weight obtained by LC/MS (all other values were from Ref. [25]). †The value was determined according to the method of Ref. [36] and the ratio was calculated with the molecular weight obtained by LC/MS.

TABLE II Pertinent NMR absorptions of the product



	Chemical shifts (ppm), multiplicity, J = Hz		
^{1}H	Product	2′-dG	8-OHdG
H-8	No signal	7.98, s	No signal
H-1′	5.88, t, J = 6.75, 6.50	6.30, t, J = 6.80, 6.90	6.24, t, J = 7.63, 6.70
H-2′	2.34, m; 2.63, m	2.51, m; 2.80, m	2.28, m; 3.07, m
H-3′	4.07, m	4.14, m	4.09, m
H-4′	4.49, m	4.63, m	4.65, m
H-5′	3.73, m	3.80, m	3.83, m
^{13}C		Chemical shifts (ppm)	
C-2	160.0	163.9	159.0
C-4	149.8	153.7	150.3
C-5	112.0	121.0	111.4
C-6	172.4	171.1	172.9
C-8	181.0	139.0	165.1
C-1′	84.8	87.3	85.1
C-2′	41.3	41.8	39.0
C-3′	74.2	74.6	74.6
C-4′	89.0	90.3	89.7
C-5′	64.7	64.9	65.0

acetate (5:2) with coloring by a reagent of diphenylamine:aniline:phosphoric acid (data not shown). The formation of 8-OHs indicated that the original product was 8-OOHdG as illustrated in Table II.

Quantitative Formation of 8-OOHdG

Figure 3A is a calibration curve for 8-OOHdG obtained using the molecular extinction coefficient $\lambda_{254}(\varepsilon) = 15,300$ in Table I. The determination limit was 3 pmol. The formation of 8-OOHdG depended on the incubation temperature (Fig. 3B) and every curve was almost linear with the incubation time. Further, the formation rates (8-OOHdG nmol min^{-1}) were linearly proportional to AAPH concentrations, indicating that the formation exhibited first-order kinetics (Fig. 3C). These results show that the present system can be useful for antioxidant assay.

Evaluation of Antioxidative Potency Using the System of 8-OOHdG Formation

We evaluated the antioxidative potency in 81 chemicals with the present system. They were dissolved in 5 µl of DMSO and added to the incubation mixture of 0.5 mM 2′-dG and 25 mM AAPH. The suppression ratios against the formation of 8-OOHdG without chemicals were determined as the antioxidative potency of the tested chemicals.

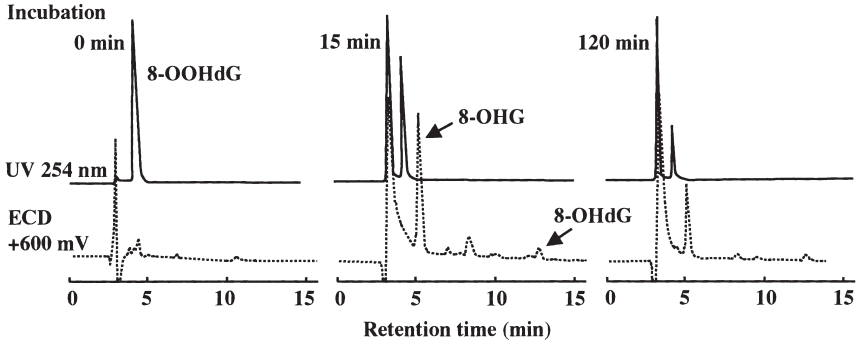


FIGURE 2 Change of 8-OOHdG to hydroxyls. The purified 8-OOHdG was incubated in 0.17N HCl at 37°C for 15 min, and then analyzed by HPLC as in Fig. 1, monitoring with UV 254 nm and electrochemically at +600 mV.

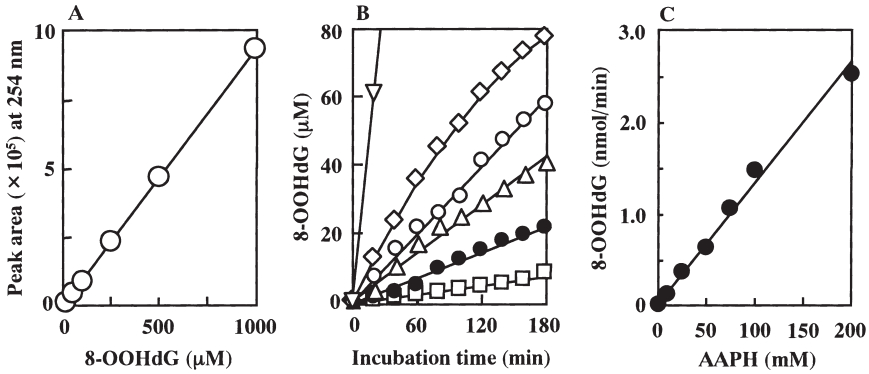


FIGURE 3 Quantitative formation of 8-OOHdG under various conditions. (A) A calibration curve for 8-OOHdG with the molecular extinction coefficient of $\lambda_{254}(\varepsilon) = 15,300$ in Table I. The determination limit was 3 pmol. (B) The formation of 8-OOHdG under various temperatures. The mixture of 0.5 mM 2′-dG and 25 mM AAPH was incubated at (□) 25, (●) 30, (△) 35, (○) 37, (◇) 40, and (▽) 50°C. (C) The formation rates of 8-OOHdG with various concentrations of AAPH. 2′-dG (0.5 mM) was incubated at 37°C with 1, 10, 25, 50, 75, 100, and 200 mM of AAPH. The amount of 8-OOHdG that formed in these incubation mixtures was determined by HPLC as in Fig. 1 every 10 min in 60 min.

The solvent DMSO had no effect on the 8-OOHdG formation.

Table III shows the antioxidative potencies of simple phenols, flavonoids, anthraquinones, other phytochemicals, food additives, and biological components, most of which occur in our diet. Almost all of them gave IC_{50} values, meaning that they were antioxidative dependent on dose. Among the simple phenols, protocatechuic acid, catechol, caffeic acid, and chlorogenic acid showed the lowest values and *ortho*-dihydroxyl products were also recognized as potent antioxidants.[41] In flavonoids, the antioxidative potencies almost coincided with their hydroxyl numbers, whereas no-hydroxyl flavone, flavanone, and chalcone were not effective and the one-hydroxyl flavonol was weaker than the polyhydroxyl flavonoids. Among anthraquinones, *ortho*-dihydroxyls showed relatively low IC_{50} values but the others had higher values or were not effective. The biological components glutathione and ascorbic acid showed high IC_{50} values, and the neurotransmitter dopamine and hormones showed relatively low values. Neither a quencher for singlet oxygen, β-carotene, nor a trapper for lipid-peroxyl radicals, α-tocopherol,[42,43] gave an IC_{50} value. On the other hand, the DPPH method was not effective for scavenging radicals or for determining the IC_{50} values of several simple phenols and flavonoids. For example, apigenin was evaluated as ineffective though it is known to be an antioxidant.[44,45]

Most of the simple phenols and flavonoids tested here are lipid-soluble. Compared to the DPPH

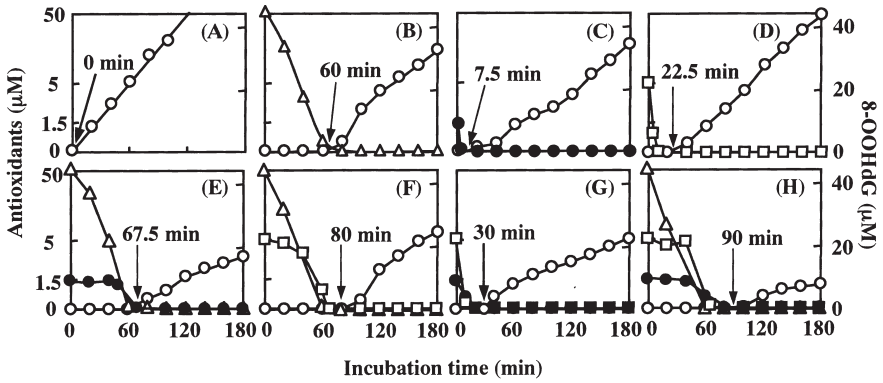


FIGURE 4 Synergism assay for the antioxidants with 8-OOHdG formation. Ascorbic acid (50 μM), quercetin (1.5 μM) and/or EGCG (5.0 μM) were added to the incubation mixture of 0.5 mM 2′-dG and 25 mM AAPH at 37°C, and the amount of 8-OOHdG (○) that formed was determined by HPLC as in Fig. 1. Simultaneously, the consumption of antioxidants was evaluated by HPLC: mobile phase for ascorbic acid (△), a 100 mM sodium phosphate buffer (pH 2.5) containing 0.1 mM ethylenediaminetetraacetate disodium salt and 5 mM tetra-*normal*--butylammonium phosphate (flow rate, 0.7 ml min^{-1}); for quercetin (●), 55% methanol in 50 mM sodium phosphate buffer (pH 3.3) containing 0.1 mM ethylenediaminetetraacetate disodium salt (flow rate, 1.0 ml min^{-1}); and for EGCG (□), 14% acetonitrile in 100 mM sodium phosphate buffer (pH 2.5) containing 0.1 mM ethylenediaminetetraacetate disodium salt (flow rate, 1.0 ml min^{-1}). Ascorbic acid and quercetin were determined with an electrochemical detector at +600 mV while EGCG was determined at +700 mV, otherwise the conditions for HPLC were as for the determination of 8-OOHdG. The panel (A), no antioxidants; (B), +ascorbic acid; (C), +quercetin; (D), +EGCG; (E), +ascorbic acid and quercetin; (F), +ascorbic acid and EGCG; (G), +quercetin and EGCG; and (H), +ascorbic acid, quercetin, and EGCG.

TABLE III Evaluation of antioxidative potency with the present system and DPPH method

Chemicals (position of substitute)	IC_{50} value (μM)* against: 8-OOHdG formation	DPPH radical
2′-dG	–	ne
Simple phenols		
1,2,4-Benzenetriol	15	19
Benzoic cid	ne	ne
Caffeic acid	6.2	26
Catechol	7.6	24
Gallic acid	16	10
Gallic acid *n*-butyl ester	15	8.5
Hydroquinone	23	25
ortho-Hydroxybenzoic acid	8.9	ne
meta-Hydroxybenzoic acid	8.9	ne
para-Hydroxybenzoic acid	6.4	ne
ortho-Hydroxycinnamic acid	12	ne
meta-Hydroxycinnamic acid	11	ne
para-Hydroxycinnamic acid	8.4	ne
Phenol	10	ne
Phloroglucinol	23	90
Protocatechuic acid	6.9	22
Pyrogallol	20	12
Resorcinol	15	ne
α-Resorcylic acid	8.8	ne
β-Resorcylic acid	10	ne
Vanillic acid	12	170
Flavonoids		
Flavones		
Flavone (none)	ne	ne
Chrysin (5,7-OH)	9.3	ne
Baicalein (5,6,7-OH)	17	25
Apigenin (5,7,4′-OH)	3.8	ne
Luteolin (5,7,3′,4′-OH)	5.0	13
Flavonols		
Flavonol (3-OH)	41	ne
Galangin (3,5,7-OH	10	68
Chrysoeriol (5,7,4′-OH,3′-OCH_3)	10	ne

TABLE III – *continued*

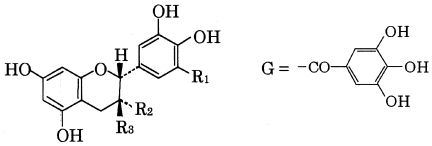
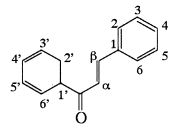
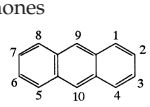
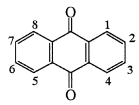
Chemicals (position of substitute)	IC_{50} value (μM)* against: 8-OOHdG formation	DPPH radical
Datiscetin (3,5,7,2′-OH)	6.9	58
Kaempferol (3,5,7,4′-OH)	1.8	24
Morin (3,5,7,2′,4′-OH)	4.1	26
Quercetin (3,5,7,3′,4′-OH)	3.8	11
Robinetin (3,7,3′,4′,5′-OH)	8.3	7.4
Isorhamnetin (3,5,7,4′-OH,3′-OCH_3)	9.8	26
Quercetagetin (3,5,6,7,3′,4′,-OH)	6.8	7.3
Myricetin (3,5,7,3′,4′,5′-OH)	5.3	8.0
Quercetine-3-L-rhamnoside (quercitrin)	1.3	18
Quercetine-3-rutinoside (rutin)	3.6	15
Flavanones (2–3 is saturated)		
Flavanone (none)	ne	ne
Naringenin (5,7,4′-OH)	3.9	ne
Hesperetin (5,7,3′-OH,4′-OCH_3)	4.8	260
(+)-Taxifolin (3,5,7,3′,4′-OH)	4.2	19
Isoflavones (B-ring binds to 3 position)		
Daidzein (7,4′-OH)	5.3	ne
Catechins		
		
(+)-Catechin (R_1 = H, R_2 = H, R_3 = OH)	7.4	16
(−)-Gallocatechin (R_1 = OH, R_2 = H, R_3 = OH)	9.3	12
(−)-Catechin gallate (R_1 = H, R_2 = H, R_3 = OG)	4.2	3.6
(−)-Gallocatechin gallate (R_1 = OH, R_2 = H, R_3 = OG)	5.1	3.2
(−)-Epicatechin (R_1 = H, R_2 = OH, R_3 = H)	3.7	4.9
(−)-Epigallocatechin (R_1 = OH, R_2 = OH, R_3 = H)	9.0	2.7
(−)-Epicatechin gallate (R_1 = H, R_2 = OG, R_3 = H)	5.3	3.4
(−)-Epigallocatechin gallate (R_1 = OH, R_2 = OG, R_3 = H)	5.8	1.6
Chalcones		
		
Chalcone (none)	ne	ne
Butein (3,4,2′,4′-OH)	5.4	17
Anthracene and anthraquinones		
 		
Anthracenes		
Anthracene (none)	93	ne
9(10H)-Anthracenone (anthrone)	ne	54
1,8-Dihydroxy-9(10H)-anthracenone (anthralin)	61	82
Anthraquinones		
Anthraquinone (none)	ne	ne
Alizarin (1,2-OH)	14	42
Quinizarin (1,4-OH)	58	130
Chrysazin (1,8-OH)	ne	ne
Rhein (1,8-OH, 3-COOH)	ne	ne
Chrysophanol (1,8-OH, 3-CH_3)	ne	ne
Purpurin (1,2,4-OH)	13	45
Emodin (1,3,8-OH, 6-CH_3)	43	ne
Other phytochemicals and food additives		
Chlorogenic acid	9.8	46
Curcumin	10	37
Sesamol	18	25

TABLE III – *continued*

Chemicals (position of substitute)	IC_{50} value (μM)* against: 8-OOHdG formation	DPPH radical
2,2,5,7,8-Pentamethyl-6-chromanol (PMC)	22	26
Trolox	23	34
Butylated hydroxyanisole (BHA)	19	46
Butylated hydroxytoluene (BHT)	52	69
tert-Butylhydroquinone (TBHQ)	29	26
Biological components		
Glutathione	52	ne
Ascorbic acid	33	45
Dopa	6.9	7.9
Dopamine	9.6	12
Adrenaline	11	10
Noradrenaline	6.5	12
β-Carotene	ne	ne
α-Tocopherol	ne	36

* Antioxidative potency was expressed as the IC_{50} value; the concentrations required to suppress the formation of 8-OOHdG by 50% in the 1-h incubation of 0.5 mM 2′-dG with 25 mM AAPH or to scavenge 50% of DPPH radicals within 5-min incubation.

method, the proposed 8-OOHdG method was able to determine the activity of lipid-soluble chemicals. The present method was also useful for evaluating the potency of water-soluble antioxidants such as catechins, Trolox, glutathione, and ascorbic acid, while the DPPH method could not detect the activity of one biologically important antioxidant, glutathione.

Determination of Synergism of Antioxidants Based on the Formation of 8-OOHdG

Ascorbic acid, quercetin, and EGCG are major dietary antioxidants from vegetables and tea in humans. Then, they were tested for synergism using concentrations of 50, 1.5, and 5.0 μM, respectively, of these antioxidants (Fig. 4), which are close to their physiological levels.[46–50] While the formation of 8-OOHdG started on incubation with 2′-dG alone (Fig. 4A), the addition of antioxidants obviously delayed the formation. The formation started at 60, 7.5, and 22.5 min into the incubation after the respective antioxidants ascorbic acid, quercetin, and EGCG, had been consumed completely (Fig. 4B–D). The presence of two antioxidants delayed the formation by the sum total of the delay caused by each antioxidant alone (Fig. 4E–G). Then, simultaneous addition of the three antioxidants prolonged the formation by 90 min (Fig. 4H). These results clearly show that the oxidative damage to 2′-dG occurs only after all antioxidants have been consumed, and that ascorbic acid, quercetin, and EGCG exhibit an additive effect on the damage.

DISCUSSION

The present study found that the molecular oxygen radicals generated from AAPH formed a novel product, an 8-OOH derivative, on 2′-dG (Table II). The formation of 8-OOHdG was linearly dependent on incubation time (Figs. 1 and 3), and so was useful for determining the antioxidative potency of various chemicals in our diet. This assay system is simple and easy, taking 15 min for the HPLC following a 1-h incubation, and is as convenient as the DPPH method. The DPPH method has been used widely, however, it employs an artificial DPPH radical making it difficult to evaluate the activity of antioxidants to prevent genetic damage. The method proposed here employs the molecular oxygen radical which is thought to be present in normal cells at high steady-state concentrations,[31] and especially in mitochondria, side reactions of the electron transport chain with molecular oxygen directly generate its radical, superoxide anion.[32,33] Also, the present method employs 2′-dG as the target and enables one to directly evaluate which antioxidants can inhibit the radical actions before the oxidative damage to 2′-dG. Additionally, this method could be used to evaluate both lipid- and water-soluble antioxidants (Table III). This method assessed that β-carotene and α-tocopherol were not antioxidative to oxygen radical, and was correct because β-carotene had been recognized to be a quencher for singlet oxygen and α-tocopherol to be a scavenger for lipid-peroxyl radicals.[42,43] So, the 8-OOHdG method is considered to be more useful than the DPPH method.

Furthermore, the present method clearly revealed an additional action of dietary antioxidants (Fig. 4). Ascorbic acid has physiological levels of around 50 μM in human plasma[46] and a few mM in cells,[47]

and quercetin, up to 1.5 μM[48,49] and EGCG 4.4 μM in plasma.[50] In Table III, their IC_{50} values for suppressing 8-OOHdG formation were 33, 3.8, and 5.8 μM, respectively. These values suggest that quercetin and EGCG alone are insufficient to prevent the genetic damage. When present together with ascorbic acid, they markedly delayed the start of genetic damage. This indicates the importance of the daily intake of antioxidants from vegetables and teas to facilitate the actions of ascorbic acid.

Thus, the method proposed here based on the formation of 8-OOHdG is very useful for evaluating the effects of the dietary antioxidants and determining the potency at which they prevent degenerative diseases including cancer associated with oxidative genetic damage.

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