In Vitro and in Vivo Studies on the Radical-Scavenging Activity of Tea

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The effects of tea (*Camellia sinensis* L.) of three types on excessive free radicals were examined utilizing spin trapping, 1,1-diphenyl-2-picrylhydrazyl radical, lipid peroxidation, and lactate dehydrogenase leakage from cultured cells. Green tea extract presented significant antiradical effects in these four assay systems, whereas oolong tea and black tea extracts showed a rather weak protective effect against free radicals. A more potent scavenger effect using cultured cells was found with a green tea tannin mixture. Similarly to the effects of the green tea tannin mixture, (−)-epigallocatechin 3-*O*-gallate, its main ingredient, had an inhibitory effect on oxidative stress-induced apoptosis. The activities of the antioxidation enzymes in rats after subtotal nephrectomy were increased, suggesting a protective action against oxidative stress. The increased levels of uremic toxins in the blood were also reduced in rats given (−)-epigallocatechin 3-*O*-gallate. These findings indicate that (−)-epigallocatechin 3-*O*-gallate helps to inhibit the progression of renal failure by scavenging radicals.

Keywords: *Tea; (−)-epigallocatechin 3-O-gallate; free radical; hydroxyl radical; apoptosis; DNA fragmentation; renal epithelial cell; nephrectomy*

INTRODUCTION

Tea (*Camellia sinensis* L.), which originated in ancient China, has been widely favored as a luxury grocery item. Recent studies have successively demonstrated the value of tea for regulation of physiological function (Okuda et al., 1983; Hikino et al., 1985; Kada et al., 1985; Fukuyo et al., 1986; Oguni et al., 1988; Ahn et al., 1991; Sakanaka et al., 1992; Yokozawa et al., 1993, 1994, 1996, 1997). Tea can be divided into three types, i.e., unfermented, semifermented, and fermented, in terms of the degree of leaf fermentation. Each type of tea has a distinct aroma, color, and flavor, which appeal to our senses of taste, smell, and sight. The physiological actions of these teas have rarely been compared, and there is quite limited information about the relationship between tea and free radicals, which mediate various diseases and pathological conditions (Miura et al., 1994; Serafini et al., 1996; Zhang et al., 1997a,b).

Attention is now being paid to xanthine oxidase and phagocytes as free radical-forming systems in the body. Once produced, free radicals induce degeneration of membrane lipid and protein, causing damage to cell membranes or degeneration of DNA and enzymes, leading to various pathological conditions (Halliwell and Gutteridge, 1984; Sies, 1991). However, organisms possess various defense mechanisms against injury by these radicals (Fujimoto, 1994).

In this connection, the present study was carried out to investigate the effect of tea and its component on injuries due to excessive free radicals, using in vitro and in vivo experimental systems.

MATERIALS AND METHODS

Tea. A 100 g amount of commercial green tea, oolong tea, or black tea was boiled gently in 1000 mL of water for 1 h. Each extract was then evaporated to dryness under reduced pressure. The yields of green tea, oolong tea, and black tea were 14.7, 16.9, and 17.5%, respectively, by weight, of the starting materials. The green tea tannin mixture employed was Sunphenon (Taiyo Kagaku Co., Yokkaichi, Japan), which was prepared from a hot-water extract of green tea, as reported previously (Sakanaka et al., 1989). It was composed mainly of (−)-epigallocatechin 3-*O*-gallate (18.0%), (−)-gallocatechin 3-*O*-gallate (11.6%), (−)-epicatechin 3-*O*-gallate (4.6%), (−)-epigallocatechin (15.0%), (+)-gallocatechin (14.8%), (−)-epicatechin (7.0%), and (+)-catechin (3.5%). A typical high-performance liquid chromatogram is illustrated in Figure 1. In addition, (−)-epigallocatechin 3-*O*-gallate was used. For purification of this component, recycling high-performance liquid chromatography was done on a JAI-LC-908 high-performance liquid chromatograph (Japan Analytical Industry Co., Tokyo, Japan) equipped with JAI RI and JAI UV detectors, operating at 280 nm, as described previously (Sakanaka et al., 1989). A prepacked PVA HP-GPC column (JAIGEL GS-320, 50 × 2 cm i.d.) was used. Methanol was used as the eluting solvent at a flow rate of 3 mL/min. The compound isolated was identified by analysis on a GC mass spectrograph (JMS-DX 303, JEOL, Tokyo, Japan) and an NMR aparatus (GSX-400, JEOL). Caffeine was purchased from Sigma Chemical Co., and theanine was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Medium and Reagents. Dulbecco's modified Eagle medium/nutrient mixture F-12 (D-MEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies, Inc. (Grand Island, NY) and Cell Culture Laboratories (Cleveland, OH), respectively. A commercial kit (lactate dehydrogenase

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S0021-8561(97)00985-0 CCC: $15.00
Published on Web 05/13/1998

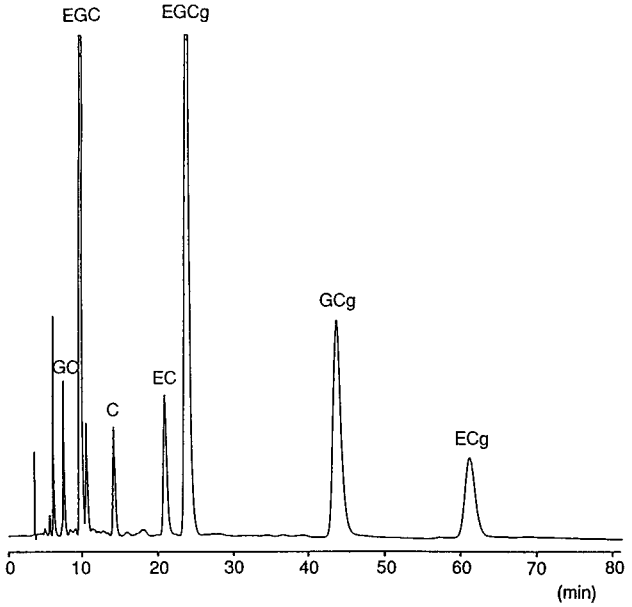

Figure 1. High-performance liquid chromatogram of green tea tannin mixture. GC = (+)-Gallocatechin, EGC = (−)-epigallocatechin, C = (+)-catechin, EC = (−)-epicatechin, EGCg = (−)-epigallocatechin 3-*O*-gallate, GCg = (+)-gallocatechin 3-*O*-gallate, ECg = (−)-epicatechin 3-*O*-gallate. Column, Develosil ODS-P-5; mobile phase, ethylacetate−acetonitrile−DMF−H_2O (3:1:15:81); flow rate, 0.5 mL/min; detection, 280 nm.

CII-Test Wako) for assaying lactate dehydrogenase (LDH) was obtained from Wako. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Aldrich Chemical Co., Milwaukee, USA.

Spin Trapping Assay. Electron spin resonance (ESR) spectroscopy combined with spin trapping using DMPO was employed to identify radical species. A 20 μL aliquot of DMPO was added to 100 μL of 100 mM homoarginine solution and 200 μL of a 10 μg/mL aqueous solution of sample, followed by stirring for 10 s. The ESR spectra of this mixture were measured with a JEOL FE-3X type spectrometer (JEOL; X-band, 100 kHz modulation) at 30 °C at 5 min after the addition of DMPO. Microwave power, modulation amplitude, and sweep time were set at 8 mW, 0.1 mT, and 0.5 min, respectively. Two peaks of external manganese dioxide appearing at $g = 1.981$ and $g = 2.034$ were used for determination of both the *g*-value and the amount of each DMPO adduct. Radical species were assigned by comparing the observed spectra with the calculated ones. The previously reported values of the hyperfine splitting constants of the DMPO adduct of a carbon-centered radical (DMPO-C) (a(N) = 1.58 mT and $a(\beta$H) = 2.42 mT), the DMPO adduct of the hydroxyl radical (DMPO-OH) (a(N) = 1.49 mT and $a(\beta$H) = 1.49 mT), and the DMPO adduct of the hydrogen radical (DMPO-H) (a(N) = 1.66 mT and $a(\beta$H) = 2.25 mT) were used for the calculation. *g*-Factors for all of the spin adducts were 2.006 (Fujita, 1987; Yokozawa et al., 1995).

Determination of the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical. In microwells, 100 μL of an aqueous solution of the sample (control:100 μL of distilled water) was added to an ethanolic solution of DPPH (60 μM) according to the method of Hatano et al. (1989). After being mixed gently and being left to stand for 30 min at room temperature, the DPPH radical was determined using a Microplate Reader, Model 3550-UV (Bio-Rad, Tokyo, Japan). The antioxidant activity of each sample was expressed in terms of IC_{50} (concentration in micrograms per milliliter required to inhibit DPPH radical formation by 50%) calculated from the log−dose inhibition curve.

Determination of Antioxidant Activities. Rat kidney was perfused with ice-cold 0.9% NaCl before homogenization. After washing with 0.9% NaCl, tissue homogenates were prepared in a ratio of 1 g of wet tissue to 9 mL of 1.15% KCl using a glass homogenizer (Ohkawa et al., 1979). The antioxidant activities were determined by quantification of thiobarbituric acid (TBA)−reactive substances using a slight modification of the method of Buege and Aust (1978). The reaction mixture was composed of 0.5 mL of kidney homogenate in 0.8 mL of phosphate buffer (50 mM, pH 7.4) and 0.3 mL of a solution of H_2O_2 (30 mM) and $FeSO_4$ (3.3 mM) with or without 0.1 mL of an aqueous solution of tea extract at a concentration of 2−50 μg/mL. The mixture was incubated at 37 °C for 20 min in a capped tube; then 4.0 mL of a stop solution, consisting of TBA/trichloroacetic acid/HCl (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl), was added to each tube, and all of the tubes were heated at 100 °C for 15 min. After a cooling period of 10 min in ice water, centrifugation was carried out at 3000 rpm for 10 min, and then determination of the supernatant was done spectrophotometrically at 535 nm. The concentration of the TBA−reactive substance generated in the mixture was calculated using an absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} L^{-1} (Wills, 1969). The antioxidant activities of tea extract were expressed in terms of IC_{50} (concentration in micrograms per milliliter required to inhibit TBA−reactive substance formation by 50%) calculated from the log−dose inhibition curve.

Experiments Using Cultured Cells. Commercially available LLC-PK_1 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air on culture plates with 5% FCS-supplemented D-MEM/F-12 medium. After confluence had been reached, the cells were seeded on fresh 96-well culture plates at 10^4 per well. The tea sample was added to the culture 2 h later, and the plates were incubated under routine conditions for 41 h. The cells were then cultured for a further 6 h under hypoxic conditions (oxygen concentration of 2% or less in a BBL Gas Pak Pouch) and reoxygenized for 1 h under ordinary culture conditions (95% air, 5% CO_2) in a CO_2 incubator. Leakage of LDH into the culture medium was assayed as an index of cytotoxicity using a commercial kit.

Experiments on Oxidative Stress-Induced Apoptosis. *(1) Cell Culture.* Commercially available LLC-PK_1 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air on culture plates with 5% FCS-supplemented D-MEM/F-12 medium. After confluence had been reached, the cells were resuspended in phosphate-buffered saline (PBS) supplemented with 50 μM H_2O_2, 0.1 mM $FeSO_4$, and the green tea sample at a concentration of 1×10^6 cells/mL, and incubated for 15 min in a CO_2 incubator. The control cells were incubated under the same conditions without the green tea sample. After 15 min of incubation, the cells were immediately centrifuged at 1500 rpm for 3 min, resuspended in fresh medium, and returned to a CO_2 incubator for a further 4 h of incubation.

(2) DNA Fragmentation Assay. According to the method of Sellins and Cohen (1987), the cells were lysed with 0.2 mL of ice-cold hypotonic lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100) and centrifuged at 16 000 rpm for 20 min to separate intact DNA from fragmented DNA. After transfer of the supernatant to a separate tube, 0.2 mL of lysis buffer was added to the pellet. To both the fragmented and intact DNA fractions, 0.2 mL of 1 M $HClO_4$ was added. After maintaining the mixture at 4 °C for 30 min, the samples were centrifuged at 16 000 rpm for 20 min and the supernatant was removed. The pellets were dissolved with 0.05 mL of 1 M $HClO_4$ and heated at 70 °C for 20 min. Then, 0.1 mL of diphenylamine solution (mixture of acetic acid, diphenylamine, and sulfuric acid, 1:15:0.15) was added and kept at 25−30 °C in a dark place overnight. The amount of DNA was determined colorimetrically at 600 nm by the method of Burton (1956). The percentage of DNA fragmentation was taken as the ratio of DNA in the supernatant to the total amount of DNA in the pellet and supernatant.

(3) DNA Fragmentation Pattern. On the basis of the method of Hockenbery et al. (1990) and Herrmann et al. (1994), the harvested cells were lysed in lysis buffer and extracted with phenol for 10 min. After centrifugation at 10 000 rpm for 2 min, the upper layer was incubated with proteinase K (50 μg/mL) at 50 °C for 60 min and then extracted with an equal volume of phenol for 10 min and centrifuged at 10 000 rpm

for 2 min. Finally, the supernatant was incubated with RNase (50 μg/mL) for 120 min at 37 °C. The DNA was extracted with an equal volume of phenol and $CHCl_3$. The DNA in the aqueous phase was precipitated by adding 2.5 volumes of EtOH and maintained at −80 °C for at least 60 min. DNA was collected by centrifugation at 15 000 rpm for 20 min, air-dried, and resuspended in TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0). Horizontal electrophoresis was performed for nearly 2 h at 100 V in a 0.75% agarose gel with TBE buffer. Equal quantities of DNA (according to determination of the optical density at 260 nm) were loaded in each lane, and Marker 4 ($\phi \times$ 174/Hae III digest, Nippon Gene) as well as Marker 6 (λ/Sty I digest, Nippon Gene) were employed as molecular weight standards. The gel was stained with ethidium bromide and photographed by UV transillumination.

Animal Experiments. *(1) Animal Preparation.* Male Wistar rats (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan) weighing 200−210 g were used. The rats underwent resection of two thirds of the left kidney and total excision of the right kidney at 10 to 14 day intervals (Platt et al., 1952; Morrison, 1966). Their blood creatinine (Cr) level was determined after recovery from the operation, and they were divided into three groups, avoiding any intergroup difference in the blood Cr level. The one group was given water, while the others were given (−)-epigallocatechin 3-*O*-gallate 2.5 and 5 mg/kg body weight/day orally for 30 consecutive days. To ensure that food consumption was almost constant among the three groups, they were raised on a commercial chow (CLEA Japan Inc., Tokyo, Japan; type CE-2) using a pair-feeding schedule. Blood samples were obtained by cardiac puncture without anesthesia, and the serum was separated immediately by centrifugation. The kidneys were subsequently extirpated from each rat following renal perfusion through the renal artery with ice-cold physiological saline.

(2) Enzyme Assays. The kidney was homogenized with a 4-fold volume of ice-cold physiological saline, and the activities of enzymes in the homogenate were determined. The activity of superoxide dismutase (SOD) was measured according to the nitrous acid method described by Elstner and Heupel (1976) and Oyanagui (1984), which is based on the inhibition of nitrite formation from hydroxylamine in the presence of superoxide (O_2^-) generators. Catalase activity was measured by following the decomposition of hydrogen peroxide (H_2O_2) directly by the decrease in extinction at 240 nm. The difference in extinction (ΔE_{240}) per unit time was used as a measure of the catalase activity (Aebi, 1974). Glutathione peroxidase (GSH-Px) activity was obtained by colorimetry of 2-nitro-5-thiobenzoic acid, a compound produced through the reaction between glutathione and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959). Protein content was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

(3) Determination of Guanidino Compounds. The serum was deproteinized by addition of TCA (final concentration, 10%). After centrifugation at 3000 rpm for 15 min, the supernatant was filtered through a 0.2 μm membrane filter and the filtrate was analyzed. Cr, methylguanidine (MG), and guanidinosuccinic acid (GSA) were measured using a Japan Spectroscopic liquid chromatograph with a step-gradient system by the method of Higashidate et al. (1984). A fluorescence spectrometer, Model FP-210 (excitation, 365 nm; emission, 495 nm; Japan Spectroscopic Co., Tokyo, Japan), was used for detection of Cr, MG, and GSA on the column.

Statistics. Data are presented as the mean ± SE of 5 determinations, where, in animal experiments, six rats were used for each experimental group. Differences among groups were analyzed by Dunnett's test. Significance was accepted at $p < 0.05$.

RESULTS

Spin Trapping. Figure 2 shows the influence of the presence of 10 μg/mL green tea extract (B), oolong tea extract (C), and black tea extract (D) on the ESR spectrum of the mixture of 100 mM homoarginine aqueous solution (100 μL) and DMPO (20 μL). In addition, the ESR spectrum obtained in the absence of any tea extract (control) is shown in Figure 2A. Although all of the peaks of DMPO adducts are not evident, DMPO-C, DMPO-OH, and DMPO-H appear in this spectrum. With regard to the spectral intensities of Mn^{2+} used as a standard, the signals from the mixture containing the green tea extract were decreased markedly in comparison with the control. Those of oolong tea and black tea also showed considerable reduction of the radical generated from homoarginine.

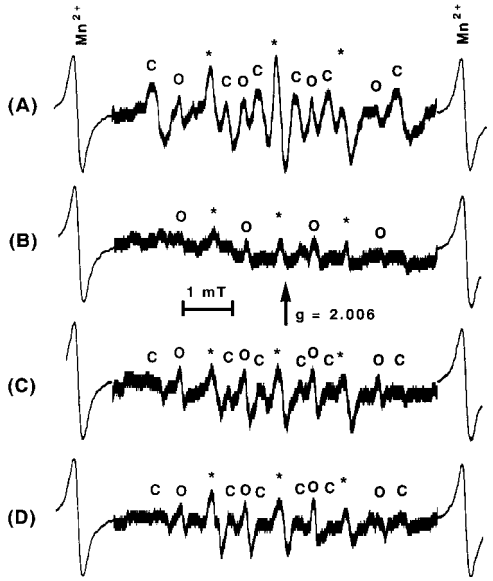

Figure 2. Influence of tea extract on the ESR spectra of a mixed solution of homoarginine and DMPO: (A) none; (B) green tea; (C) oolong tea; (D) black tea. C = DMPO-C, O = DMPO-OH, asterisk = DMPO-H.

Table 1. IC_{50} Values of Tea against the DPPH Radical

material	IC_{50} (μg/mL)
green tea	4.14 ± 1.00
oolong tea	47.12 ± 0.99
black tea	27.02 ± 0.96

Table 2. IC_{50} Values of Tea on Lipid Peroxidation Induced by H_2O_2 and Fe^{2+} in Renal Homogenate

material	IC_{50} (μg/mL)
green tea	7.22 ± 0.39
oolong tea	12.10 ± 0.81
black tea	10.55 ± 0.56

DPPH Radical. As shown in Table 1, it was found that the green tea extract had very significant scavenging activity on the DPPH radical, having 50% inhibitory activity at a low concentration of 4.14 μg/mL. On the other hand, the other tea extracts seemed to have a relatively weak effect, the IC_{50} value being 47.12 μg/mL for oolong tea and 27.02 μg/mL for black tea.

Lipid Peroxidation. All of the tea extracts tested showed distinctive inhibition of TBA−reactive substance formation in the reaction mixture treated with H_2O_2 and Fe^{2+}. As shown in Table 2, green tea extract showed 50% inhibition at a concentration of 7.22 μg/mL, whereas oolong tea and black tea extracts, with a large IC_{50}, showed a rather weak protective effect against lipid peroxidation.

LDH Leakage from Cultured Cells. As the result of reoxygenation after 6 h of hypoxic culture, the leakage

Table 3. Effect of Tea on LDH Leakage from LLC-PK$_1$ Cells Subjected to Hypoxia-Reoxygenation (Statistical Significance: $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$ vs Value for None)

material	concn (μg/mL)	LDH activity (mIU/mL)	material	concn (μg/mL)	LDH activity (mIU/mL)
green tea	1.25	141.1 ± 5.4^b	oolong tea	25	143.9 ± 6.8^a
	2.5	134.2 ± 6.7^c		50	138.6 ± 7.6^b
	12.5	134.2 ± 7.3^c	black tea	1.25	147.6 ± 6.5
	25	128.9 ± 6.8^c		2.5	144.5 ± 6.8^a
	50	106.4 ± 7.9^c		12.5	142.3 ± 7.2^b
oolong tea	1.25	156.3 ± 8.3		25	137.9 ± 7.0^c
	2.5	153.1 ± 7.9		50	128.4 ± 6.8^c
	12.5	150.1 ± 7.3	control		158.8 ± 5.3

Table 4. Effect of Green Tea Component on LDH Leakage from LLC-PK$_1$ Cells Subjected to Hypoxia-Reoxygenation (Statistical Significance: $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$ vs Value for None)

material	concn (μg/mL)	LDH activity (mIU/mL)	material	concn (μg/mL)	LDH activity (mIU/mL)
tannin	0.25	134.1 ± 8.4^b	caffeine	5	133.2 ± 6.6
mixture	0.5	112.4 ± 6.4^c		10	135.6 ± 8.6
	2.5	110.4 ± 5.0^c	theanine	0.25	144.2 ± 6.5
	5	92.9 ± 3.8^c		0.5	143.7 ± 8.4
	10	82.2 ± 2.4^c		2.5	137.3 ± 7.2
caffeine	0.25	146.8 ± 9.3		5	132.9 ± 6.8^a
	0.5	147.5 ± 7.8		10	129.3 ± 6.0^b
	2.5	140.0 ± 7.6	control		147.4 ± 4.2

of LDH from the LLC-PK$_1$ cells into the culture medium increased markedly to about 160 mIU/mL, being nearly 1.5-fold that observed under ordinary culture conditions (95% air, 5% CO_2). On the other hand, when the green tea extract was added to the medium to give a final concentration of 1.25 μg/mL, leakage of the enzyme due to reoxygenation was significantly reduced to 141.1 ± 5.4 mIU/mL, as shown in Table 3. This suppressive effect increased with increasing concentration of the green tea extract. A similar trend was found in the black tea extract group, although the inhibitory effect was weaker than that of green tea extract. Treatment of the cells with oolong tea extract limited this leakage marked, only 25 and 50 μg/mL appearing in the medium.

As shown in Table 4, the green tea tannin mixture proved to be the most potent, showing significant inhibition at a concentration of 0.25 μg/mL and at a 44% lower level at a concentration of 10 μg/mL when compared with the value in the absence of the tannin mixture. However, caffeine was found to lack such an effect, while theanine significantly inhibited the leakage of LDH at concentrations of 5 and 10 μg/mL, as shown in Table 4.

DNA Fragmentation. As shown in Table 5, DNA of LLC-PK$_1$ cells was fragmented into lower-molecular-weight molecules even after a very short exposure time, causing a great increase in the proportion of free low-molecular-weight DNA per total amount of DNA. Analysis of the agarose gel electrophoresis pattern revealed a ladder, which was absent in cells not subjected to the Fenton reaction, indicating that oxidative stress induced apoptosis (Figure 3). On the other hand, it was confirmed in terms of the rate of DNA fragmentation that, in cells subjected to the Fenton reaction in the presence of a 30 μg/mL green tea tannin mixture, DNA fragmentation was less severe and that when the concentration of tannin mixture was increased to 60 μg/mL, it was suppressed to an extent close to that in cells that were not subjected to the Fenton reaction (Table 5). The data

Table 5. Effect of Green Tea Components on DNA Fragmentation (Statistical Significance: $^a p < 0.05$, $^b p < 0.001$ vs Value for None; $^c p < 0.05$, $^d p < 0.001$ vs Value for $H_2O_2 + Fe^{2+}$ Only)

addition	fragmentation rate (%)
none	22.2 ± 2.9
$H_2O_2 + Fe^{2+}$	69.0 ± 5.8^b
$H_2O_2 + Fe^{2+}$ + tannin mixture (30 μg/mL)	45.1 ± 3.9^{b,d}
$H_2O_2 + Fe^{2+}$ + tannin mixture (60 μg/mL)	29.3 ± 3.4^d
$H_2O_2 + Fe^{2+}$ + caffeine (30 μg/mL)	63.9 ± 5.4^b
$H_2O_2 + Fe^{2+}$ + caffeine (60 μg/mL)	59.6 ± 6.6^b
$H_2O_2 + Fe^{2+}$ + theanine (30 μg/mL)	59.8 ± 6.3^b
$H_2O_2 + Fe^{2+}$ + theanine (60 μg/mL)	51.5 ± 6.1^{b,d}
$H_2O_2 + Fe^{2+}$ + (−)-epigallocatechin 3-*O*-gallate (2.5 μg/mL)	40.1 ± 3.4^{b,d}
$H_2O_2 + Fe^{2+}$ + (−)-epigallocatechin 3-*O*-gallate (5 μg/mL)	32.1 ± 2.5^{a,d}

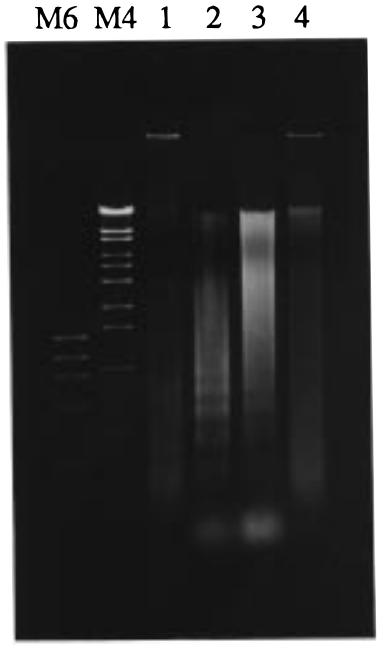

Figure 3. Agarose gel electrophoresis of DNA: Lane 1, none; lane 2, $H_2O_2 + Fe^{2+}$; lane 3, $H_2O_2 + Fe^{2+}$ + (−)-epigallocatechin 3-*O*-gallate (2.5 μg/mL); lane 4, $H_2O_2 + Fe^{2+}$ + (−)-epigallocatechin 3-*O*-gallate (5 μg/mL); M4, marker 4; M6, marker 6.

for (−)-epigallocatechin 3-*O*-gallate showed a further effect on the rate of DNA fragmentation and electrophoresis patterns (Table 5 and Figure 3). However, similar to the results under hypoxia/reoxygenation, caffeine showed no inhibitory effect like that observed with the tannin mixture. Theanine caused significant inhibition of DNA fragmentation at a concentration of 60 μg/mL, as shown in Table 5.

Animal Experiments. *(1) Enzyme Activities.* Table 6 shows the activities of reactive oxygen species-scavenging enzymes. In comparison with normal rats, enzyme activities were significantly decreased in rats given no (−)-epigallocatechin 3-*O*-gallate, the value being 51% lower for SOD activity, 35% lower for catalase activity, and 13% lower for GSH-Px activity. The activities of both SOD and catalase, however, were higher in rats given (−)-epigallocatechin 3-*O*-gallate at both the 2.5 and 5 mg dosage level for 30 days after nephrectomy. As shown in Table 6, the SOD activity was increased significantly by 33 and 63% in rats given

Table 6. Effect of (−)-Epigallocatechin 3-*O*-Gallate on the Activities of Reactive Oxygen Species-Scavenging Enzymes in Rats after Nephrectomy (Statistical Significance: [a]$p < 0.01$, [b]$p < 0.001$ vs Normal Rats; [c]$p < 0.001$ vs Nephrectomized Control Rats)

group	dose (mg/kg of BW/day)	SOD (U/mg of protein)	catalase (U/mg of protein)	GSH-Px (U/mg of protein)
nephrectomized rats				
control		8.90 ± 0.32^{b}	135.6 ± 11.2^{b}	73.89 ± 4.91^{a}
(−)-epigallocatechin 3-*O*-gallate	2.5	$11.86 \pm 0.54^{b,c}$	$172.1 \pm 10.3^{b,c}$	75.53 ± 4.28^{a}
(−)-epigallocatechin 3-*O*-gallate	5	$14.49 \pm 0.70^{b,c}$	198.3 ± 9.4^{c}	77.98 ± 3.59
normal rats		18.15 ± 1.23	210.0 ± 9.3	85.00 ± 3.02

Table 7. Effect of (−)-Epigallocatechin 3-*O*-Gallate on the Serum Guanidino Compounds (ND, Not Detectable; Statistical Significance: [a]$p < 0.001$ vs Normal Rats; [b]$p < 0.001$ vs Nephrectomized Control Rats)

group	dose (mg/kg of BW/day)	Cr (mg/dL)	MG (μg/dL)	GSA (μg/dL)
nephrectomized rats				
control		2.16 ± 0.13^{a}	3.38 ± 0.18	51.02 ± 4.10
(−)-epigallocatechin 3-*O*-gallate	2.5	$1.53 \pm 0.10^{a,b}$	2.68 ± 0.15^{b}	35.21 ± 3.16^{b}
(−)-epigallocatechin 3-*O*-gallate	5	$1.20 \pm 0.09^{a,b}$	1.89 ± 0.13^{b}	29.82 ± 2.47^{b}
normal rats		0.67 ± 0.04	ND	ND

2.5 and 5 mg of (−)-epigallocatechin 3-*O*-gallate, respectively. Similarly, (−)-epigallocatechin 3-*O*-gallate significantly increased the catalase activity from 135.6 to 172.1 U/mg of protein at the 2.5 mg level (a 27% change, $p < 0.001$) and from 135.6 to 198.3 U/mg of protein at the 5 mg level (a 46% change, $p < 0.001$). The GSH-Px activity was almost the same as that in the controls. There were no significant differences in protein level between the control and (−)-epigallocatechin 3-*O*-gallate-treated groups.

(2) Guanidino Compounds. Table 7 shows the effect of (−)-epigallocatechin 3-*O*-gallate on parameters of guanidino compounds after administration of an oral dose. The Cr level in nephrectomized control rats was increased significantly compared to the level in normal rats. The levels of serum MG and GSA were 3.38 and 51.02 μg/dL, respectively, in rats that had undergone nephrectomy, whereas MG and GSA were not detected in the sera of normal rats. In contrast, oral administration of 2.5 mg of (−)-epigallocatechin 3-*O*-gallate caused a significant decrease in the levels of Cr, MG, and GSA compared with those in nephrectomized control rats. A further increase in the dose to 5 mg produced a further decrease in these guanidino compounds.

DISCUSSION

Various substances in the body possess a guanidine group in their structures. Mori et al. (1980) and Yokoi et al. (1984) have reported that intraventricular injection of α-guanidinoglutaric acid or homoarginine induces generalized seizures in rats, suggesting involvement of the formation of $^{\bullet}O_2H$ and $^{\bullet}OH$ radicals. On the other hand, as shown in Figure 2, the ESR spectrum of homoarginine solution shows the 1:2:2:1 quartet pattern peculiar to DMPO-OH. There are many possible mechanisms by which $^{\bullet}OH$ radicals are generated from an aqueous solution of a guanidine compound. In general, there is equilibrium between guanidine ions and electrons in a guanidine solution. These electrons are considered to react with oxygen molecules in the solution to generate O_2^-, resulting in formation of $^{\bullet}OH$ through the Haber−Weiss reaction (Bors et al., 1979). However, in the presence of green tea extract at a concentration of 2 μg, the production of $^{\bullet}OH$ was markedly low. A similar $^{\bullet}OH$ radical-eliminating action was noted for oolong tea and black tea, although it was less potent than green tea.

The reactivity of $^{\bullet}OH$ is particularly high among various different radicals. On the other hand, the DPPH radical is stable in ethanolic solution for more than 60 min (Hatano et al., 1989). Because of this, we used this system for assessing the radical-scavenger activity of three types of tea extract. As a result, the descending order of the scavenger activity was green tea > black tea > oolong tea, the same as the result obtained by the spin trap method.

Lipid peroxides produced from unsaturated fatty acid by radicals have histotoxicity by themselves and also increase the production of free radicals in a manner of chain reaction (Fong et al., 1973; Weiss et al., 1977). On the basis of these findings, we added various concentrations of tea extracts to the incubation medium of an experimental system in which the Fenton reaction was induced in a kidney homogenate in the presence of H_2O_2 and Fe^{2+} and obtained their IC_{50} values. The descending order of IC_{50} was green tea > black tea > oolong tea; thus green tea extract was proven to have high activity, suggesting its scavenger action on $^{\bullet}OH$ produced from H_2O_2 and Fe^{2+}.

Active oxygen species currently known to be produced in the body include O_2^-, H_2O_2, $^{\bullet}OH$, $^1O_2$, and LOOH. Although O_2^- does not have the strongest toxicity among these species, it plays an important role as an initiator in the generation of various types of active oxygen (McCord, 1985). Therefore, it is generally considered that above all, elimination of O_2^-, which is the origin of various other types of active oxygen in the body, is crucial. With regard to the source of active oxygen in ischemia-reperfusion injury, Saugstad and Aasen (1980) reported that ATP molecules produced in ischemia are decomposed to adenosine and hypoxanthine, with conversion of xanthine dehydrogenase to xanthine oxidase, and that a large amount of O_2^- is produced in the presence of the increased hypoxanthine and xanthine oxidase when oxygen is supplied by blood flow in reperfusion. In the present study, we used the swine kidney-derived cultured epithelial cell line LLC-PK_1 (Gastraunthaler, 1988), which has features resembling those of proximal uriniferous tubules, the site of severe injury in ischemic acute renal failure, in order to assess renal ischemic-reperfusion injury in vitro. When these cells were reoxygenated after incubating them under hypoxic conditions for 6 h, LDH leakage into the culture medium increased markedly to reach a level of about

160 mIU/mL. On the other hand, when green tea extract was added to the medium in advance to a final concentration of 1.25 μg/mL, leakage of the enzyme due to reoxygenation was suppressed to a significant degree. Higher concentrations of green tea extract enhanced the effect. In cases of black tea extract and oolong tea extract, significant suppression of LDH leakage into the medium was not obtained until double and 20-fold the corresponding concentration of green tea extract, respectively, was added to the medium.

In any of the four experimental systems examined, the radical-scavenger activity of green tea extract was higher than that of black tea and oolong tea extract. A similar, and more potent, scavenger effect using LLC-PK_1 cells was found with a tannin mixture. However, no such effect was found with caffeine, which is a component having an analeptic action proper to tea leaves, while theanine, a component which contributes to the "tastefulness" of green tea, was associated with only a slight leakage of LDH.

Using LLC-PK_1 cells, Yonehana and Gemba (1994) have demonstrated that the intracellular antioxidant glutathione decreases significantly under hypoxic conditions. Snowdowne et al. (1985) and Kribben et al. (1994) have also reported that hypoxia causes the intracellular Ca concentration to increase in another kidney-derived culture cell line, LLC-MK_2, or in isolated uriniferous tubules. On the other hand, with regard to cell injury due to reoxygenation, Paller et al. proposed in 1984 the new theory that active oxygen is involved in the pathogenesis of renal ischemic-reperfusion injury, and this had a considerable influence in this field of science. Since then, a close relationship between ischemic-reperfusion injury in various organs and their diseases has become gradually apparent. In the proximal tubule-like LLC-PK_1 cells used in the present study, leakage of LDH was suppressed when DMSO, an $^{\bullet}OH$ scavenger, was added to the medium prior to the start of culture (data not shown), suggesting that free radicals produced by renal epithelial cells through hypoxia and reoxygenation are responsible for the cell injury. This result is consistent with the finding of Paller and Neumann (1991) that renal epithelial cells produce free radicals in primary renal cell culture. The fact that this type of cell injury was suppressed by green tea extract, and more potently by tannin, a component of green tea extract, is in vitro evidence for the direct effect of green tea tannin on renal cells, corroborating our previous finding that oral administration of green tea tannin ameliorated renal failure in rats under oxidative stress (Yokozawa et al., 1996). The viability of the renal cells, however, was not affected by this action. On the other hand, unlike green tea, oolong tea is produced through a process of sun-drying and indoor drying including turning over (stirring of tea levels while swaying them), which aids the serial steps of enzyme reaction to proceed slowly and steadily, to produce the necessary changes in the tea components. In the present study, oolong tea proved to have the lowest activity among the three types of tea examined. The radical-scavenger activity of black tea, which is processed by fermentation after milling of the cells, was lower than that of green tea, but higher than that of oolong tea, demonstrating at the cellular level that the difference in the fermentation process affects the radical-scavenger activity of tea.

On the other hand, in cells of the living body, H_2O_2 is produced by disproportionation of O_2^-, and the H_2O_2 produced is converted to a more potent oxidant, $^{\bullet}OH$. Much attention is now being focused on the cytotoxicity of these active oxygen free radicals as the cause of various pathological conditions. Using thymocytes, Forrest et al. (1994) revealed that active oxygen induces apoptosis and that such apoptosis is suppressed by a water-soluble vitamin derivative (Trolox). In addition, Ueda et al. (1995) have found that apoptosis is induced following activation of endonuclease when LLC-PK_1 cells are exposed to H_2O_2. In the present study using an experimental system in which LLC-PK_1 cells were exposed to $^{\bullet}OH$ produced from H_2O_2 and $FeSO_4$ (Fenton reaction), DNA of LLC-PK_1 cells were fragmented into lower-molecular-weight molecules even after a very limited exposure time, causing a great increase in the proportion of free low-molecular-weight DNA per total amount of DNA. Analysis of the agarose gel electrophoretic pattern revealed a ladder, which was absent in cells that were not subjected to the Fenton reaction, indicating that oxidative stress induced apoptosis. However, when cells were subjected to the Fenton reaction in the presence of a tannin mixture at 30 μg/mL, the degree of DNA fragmentation was lowered. In the presence of the tannin mixture at 60 μg/mL, DNA fragmentation was suppressed to a degree approximating that in cells that were not subjected to the Fenton reaction. When 2.5 μg/mL of (−)-epigallocatechin 3-*O*-gallate, the major ingredient of the tannin mixture, was added to the culture medium, the degree of apoptosis was lowered, as confirmed by the rate of DNA fragmentation and the electrophoretic pattern. The mechanism of such effect may be in two ways: one is metal chelation because of the properties of the phenol−hydroxyl group; the other may be the reduction property of double bonds in aromatic rings.

To cope with oxidative stress, organisms have graded antioxidant mechanisms inside and outside the cell. The results of the present study showed that (−)-epigallocatechin 3-*O*-gallate influenced the activity of radical-scavenger enzymes in subtotally nephrectomized rats, leading to increased activity of SOD, an enzyme which catalyzes disproportionation of O_2^- into H_2O_2. Moreover, (−)-epigallocatechin 3-*O*-gallate induced an increase in the activity of catalase, an enzyme which specifically eliminates H_2O_2 and suppresses the formation of $^{\bullet}OH$ and OCl^-. On the other hand, there was no change in GSH-Px, which, like catalase, is an enzyme that helps eliminate H_2O_2 and is localized in the matrix of mitochondria. This suggests that the site of action of (−)-epigallocatechin 3-*O*-gallate is the peroxisome. Peroxisomes in the kidney of the rat contain D-amino acid oxidase and flavin enzymes, such as α-hydroxy-acetic acid oxidase, and Cu enzymes such as uricooxidase, and produce H_2O_2 through the oxidation of respective substrates. Catalase works to detoxify this H_2O_2 and to cleave long-chain fatty acids by using H_2O_2 (a kind of β-oxidation) (Nakano, 1991). The fact that tannin contained in a luxury grocery item, tea, increases the activity of this enzyme indicates the possible presence of a promising novel functional substance. In addition, Cr, MG, and GSA, which are known to accumulate in blood with the progression of renal failure (Yokozawa et al., 1986), were also decreased in rats given (−)-epigallocatechin 3-*O*-gallate, indicating that elimination of free radicals leads to relief of renal disorder. Thus, it is apparent that free radicals play an important role in the progression of renal failure.

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Received for review November 18, 1997. Revised manuscript received March 23, 1998. Accepted March 26, 1998.

JF970985C