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Oxidative DNA damage by Ethanol Extract of Green Tea

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ABSTRACT : Green tea and their major constituents such as catechins are famous materials for their anti-oxidative and anti-carcinogenic activity, but many compounds with reducing power can promote the oxidation in their oxidized form or in the presence of metal ion. We investigated the pro-oxidative effect of the ethanol extract equivalent up to 30 mg of dried weight of green tea leaves in four *in vitro* systems which could be used for detecting DNA damage. Although ethanol extract of green tea did not show significant mutagenicity in *Salmonella typhimurium* TA102, which is sensitive strain to oxidative stress, it degraded deoxyribose extensively in the presence of $FeCl_3$-EDTA complex, promoted 8-oxoguanine formation in the live bacteria cell, *Salmonella typhimurium* TA104, and cleaved super coiled DNA strand with the help of copper ion. It suggested that green tea, famous anti-oxidative material, can be pro-oxidant according to the condition of extraction or metal existence.

Key words : **Green tea, 8-oxoguanine, oxidative stress, DNA damage**

Introduction

Green tea is one of the most popular commodities in oriental society. It contains various polyphenols like catechins and other flavonoids like quercetin (14-23 mg/l), kaempferol (9-15 mg/l), and myricetin (5-12 mg/l) (Hertog *et al.*, 1993). Recently many researches have been conducted to show the anti-oxidative effect of green tea components. Green tea polyphenol was reported to inhibit the DNA strand breakage of the lung cell and reduce the lipid peroxidation when pretreated or treated with oxidant (Leanderson *et al.*, 1997). Catechin and quercetin were reported to prevent lipid peroxidation on iron-loaded hepatocyte culture (Morel *et al.*, 1993) and caffeine also known to contribute to the anti-carcinogenic activity of green tea on UVB light treated SKH-1 mice (Huang *et al.*, 1997). Particularly, green tea catechins were studied thoroughly and the mechanism of its antioxidant action on lipid peroxidation and that of LDL lipid peroxidation was partially identified (Valcic *et al.*, 1999).

However, most studies on green tea concerned the anti-oxidative effect 'on the lipid peroxidation' and 'in the severe stressful situation'. Previous researches also focused on the effect of purified component. In the other biomolecules like protein or DNA, other than lipid system, the same compound or whole green tea can promote the production of reactive oxygen species with the help of transition metal like copper. Although it once acts as the reductant, the oxidized products are willing to be reduced, so it can be oxidant, oppositely. Epicatechin and other flavonoids were reported to be able to induce the DNA strand breakage and produce the reactive oxygen species in the presence of Cu (II) (Chrisey *et al*, 1988; Ahmad *et al*, 1992; Fazal *et al*, 1990). Copper is closely associated with chromosome and DNA bases, so it is possible that such compounds can oxidize the DNA through a copper-redox cycle mechanism (Li and Trush, 1994).

Anti-oxidative capacity of some green tea component is very important. However, if there are other substances which block the activity of other compound or do more harmful effect, it can be dangerous situation. People have been consumed the tea from the very ancient time in the form of water extract, so the resulted tea can be a complex mixture, of which the effect would not be equivalent to that of a purified material. Furthermore overemphasis on the effect of green tea could be resulted in the new product using the whole tea material, of which either positive or negative effect is not studied sufficiently.

In our previous study (not published), aqueous extract of green tea didn't show any particular pro-oxidative effect. So, in this study we experimented with ethanol extract of green tea (EGT) and considered its DNA oxidation effects.

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At first, reducing capacity via Fenton reaction was tested *in vitro* assay. Next, oxidized DNA bases marked with 8-oxoguanine in the bacteria cell were quantitated, and mutagenicity on the *Salmonella typhimurium* TA 102 which is sensitive to oxidative stress was tested. Then, by testing the supercoiled DNA breakage *in vitro* assay, participant of copper ion was determined.

Materials and Methods

Chemicals and samples

Deoxyribose, ascorbic acid, ferric chloride, EDTA, 8-hydroxy-2'-deoxyguanosine and 6-azathymine were obtained from Sigma Chemical Co. (St. Louis, MO); Tryptic soy broth was from Difco (Detroit, MI); BSTFA was from Pierce Chemical Co. (Rockford, IL); Wizard™ DNA purification kit was from Promega (Madison, WI); ϕX-174 RF DNA was from Gibco (Gaithersburg, MD); *Salmonella typhimurium* TA 102 and 104 were provided by Ph.D. Surh Y.J. of the department of medicine, Seoul National University.

Green tea was purchased in commercial dried form (Pacific Co. Korea). Dried sample (3 g) was extracted three times with ethanol using microwave extraction system (MES-100, CEM). The sample was filtered after each extraction. Ethanol extract was concentrated to 10 ml using a vacuum rotary evaporator (Heidolph). When extract was used, except 8-oxoguanine measurement, it was dried with N_2 gas and redissolved with DMSO.

Deoxyribose degradation test

Reaction mixture was made according to a protocol described by Aruoma *et al.* (1991). In short, it contains 2.8 mM deoxyribose, 20 uM ferric chloride, 100 uM EDTA, 1.4 mM hydrogen peroxide, and 12.6 mg (in dry weight of green tea leaves) of EGT and adjusted to 1 ml with phosphate buffer (10 mM, pH 7.4). In control, there was no EGT, and in positive control, ascorbic acid replaced the EGT. Prepared mixture was incubated at 37°C for 1 hour. After incubation, reaction was stopped by 500 ul of 2% (w/v) TBA and 250 ul of 11.2% (w/v) TCA, and maintained at 100°C for 15minutes. Absorbance at 532 nm of degraded deoxyribose, TBARS, was measured with spectrophotometer (DU 600, Beckman).

Quantification of 8-hydroxyguanine by GC-MS

Hydroxylation of guanine base was measured using *S. typhimurium* strain TA 104 that is sensitive to oxidative stress but has low background level. Samples and bacteria were treated using the method described by Abu-Shakra (1997). Bacteria cells were grown overnight in tryptic soy broth to over $1–2\times10^9$ cells/ml. Cells were harvested by centrifugation at 4,000 rpm for 15 minutes. EGT (30 mg in dry weight of green tea leaves) and aliquot (10 ml) of cells resuspended in sodium phosphate buffer (0.1 M, pH 7.4) were vortexed and incubated in the water bath for 30 minutes. Incubation was performed at 4°C to prevent repair. After incubation, the cell was centrifuged and washed twice with cold sterile water. The DNA was isolated from the cell pellet using Wizard™ DNA purification kit (Promega), avoiding the use of organic solvent like phenol which can contribute to artificial hydroxylation.

Isolated DNA (about 150 ug) and internal standard, 6-azathymine, were lyophilized and hydrolyzed to free base with 88% formic acid at 150°C for 30 minutes. Then the DNA was lyophilized again and derivatized with a mixture of BSTFA and acetonitrile (2 : 1) at 120°C for 30 minutes. Before the derivatization, the mixture was purged with inactive nitrogen gas to prevent artificial hydroxylation (Collins *et al.*, 1997). Derivatized hydrolysate of DNA was analyzed with SPB-5 fused silica capillary column (supelco, 12 m×0.20 mm, 0.33 um thickness), gas chromatography (Hewlett Packard, GC 6890) and mass spectrometry (Hewlett Packard, MS 5973). Injection port, ion source and interface were kept at 250°C. The column temperature was increased from 150°C to 250°C at a rate of 8°C/min after 2 minutes at 150°C. Helium was used as carrier gas at inlet pressure of 40 kPa. 4 ul of sample was injected as 15 : 1 split mode, and detected with selected ion monitoring (SIM). Up to 8 minutes, ion group of m/z 256, 271 was monitored for 6-azathymine. After 8 minutes, ion group of m/z 440, 455 was monitoring for 8-oxoguanine. Integrated peak area was converted to relative molecular response factor (RMRF) compared with the area of internal standard and the mass of DNA.

Mutagenicity test

The mutagenicity of EGT was determined in *Salmonella typhimurium* TA 102. EGT was redissolved in DMSO, and treated as following 'the standard assay without preincubation' excluding S-9 mixture (Levin D.E. *et al.*, 1984). In short, 0, 6.3, 12.6 or 25.2 mg of EGT in dry

weight of green tea leaves, 100 ul bacteria culture, and 2 ml soft agar were briefly mixed and poured into minimal agar plate. After 48-hour incubation in 37°C, the revertants were counted.

DNA strand breaks

35 ug ϕX-174 form I supercoiled DNA was mixed with 12.6 mg/ml EGT in dry weight of green tea leaves, and with or without 20 uM $CuSO_4$ in phosphate buffer (0.1 M, pH 7.4). The control mixture contains DMSO in the same volume with sample stock. The mixture was incubated for 1hour, at 37°C, and then electrophoresed in 1% agarose gel, at 80 V for 4 hours. After stained with ethidium bromide, supercoiled, linear or open circular form were defined under UV lamp and photographed.

Results

Deoxyribose degradation effect

To assess the reducing capacity and the effect on the deoxyribose, which is constituent of DNA, EGT was treated with ferric ion and hydrogen peroxide. Ascorbic acid, powerful reducing agent, can reduce Fe (III) to Fe (II) and promote the generation of hydroxyl radical from hydrogen peroxide. So produced radical could degrade the deoxyribose to form the malondialdehyde type product that generate pink chromogen on heating with TBA. Like the reducing activity of ascorbic acid, EGT could enable the prolonged oxidation of deoxyribose. It promoted the deoxyribose degradation up to 2.5 times compared with control without EGT (Table 1). This showed statistically very significant difference ($p = 5.71 \times 10^{-7}$).

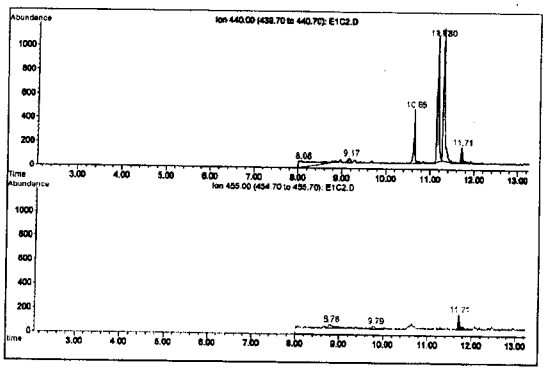
Formation of oxidative DNA base adduct in living cell

To make the similar condition with the living organism, live bacteria cells, which is sensitive to oxidative stress were incubated with EGT, and the bacteria DNA was analyzed to show the oxidative DNA base modification marked with 8-oxoguanine. In Fig. 1, selected ion (m/z 440 and 455) chromatograms of 8-oxoguanine were shown. Upper panel (a) is the chromatogram from the bacteria which was incubated without EGT. The abundance of the peak painted black is very low, about 200. Lower panel (b) is from the bacteria, incubated with EGT for 30 minutes, and the black peak abundance is much higher than that of panel

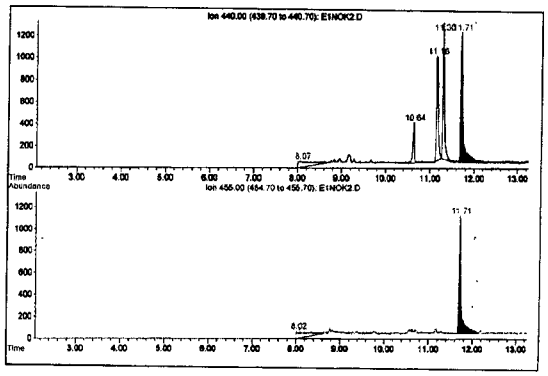
Table 1. Deoxyribose degradation capacity of ethanol extract of green tea

	OD (532 nm)	Significance
Control	0.4040 ± 0.0159	
Ascorbic acid	2.1244 ± 0.0442	$P < 0.001$
Ethanol extract of green tea	0.9932 ± 0.0083	$P < 0.001$

In control reaction mixture, 2.8 mM deoxyribose was incubated with 1.4 mM hydrogen peroxide, 20 uM ferric chloride chelated with 100 uM EDTA in phosphate buffer (10 mM, pH 7.4) for 1 hr, at 37°C. In the ascorbic acid reaction mixture 100 uM ascorbic acid was added. In the last reaction mixture, 12.6 mg/ml (dry weight of green tea leaves) of ethanol extract of green tea was added after evaporating the ethanol. After the reactions were stopped by TCA and boiled with TBA, absorbance of TBARS was measured at 532 nm. All reactions were triplicated, and the values are mean ± S.D.. Difference with control is represented as P-value (Student's *t*-test).



(a)



(b)

Fig. 1. SIM Chromatogram of 8-oxoguanine from Salmonella typhimurium TA 104
Not treated (a) and treated with ethanol extract of green tea (b) at 4°C for 30min. 8-oxoguanine was acquired from isolated DNA of treated bacteria cells by hydrolysis and derivatization with BSTFA. Peak was determined as what has similar intensity of m/z 440, 455 ions (peaks painted black). RMRF of (a) and (b) were 0.0114 and 0.0423 respectively.

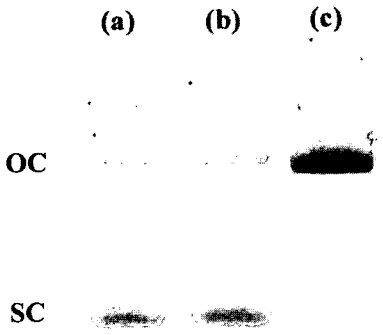


Fig. 2. Strand scission of ϕX174 supercoiled DNA by ethanol extract of green tea.
35 ug of supercoiled ϕX174 DNA was incubated for 30 min at 37°C with 10 ul DMSO (a), and 12.6 mg/ml ethanol extract of green tea (in dry weight of green tea leaves) dissolved in DMSO (b). 20 uM CuSo4 and ethanol extract of green tea extract were treated additionally (c).
OS : open circular form SC : super coiled from

(a). Peak area was adjusted with the amount of used DNA and the internal standard. The values of relative molecular response factor (RMRF), were 0.0114 and 0.0423 respectively.

Mutagenicity in Ames test

Although EGT showed some oxidative DNA damage, it didn't show mutagenicity in oxidative stress-sensitive bacteria cell, *Salmonella* typhimurium TA 102. As incubated sample dose increased to 25.2 mg in dry weight of green tea leaves, reverted colony numbers showed neither significant increase nor decrease (data not shown).

DNA strand breaking effect in the presence of metal ion

To confirm the role of ethanol extract of green tea on DNA damage, supercoiled DNA (∅X 174 form I) was incubated with the sample in the absence or presence of Cu (II). As shown in Fig. 2, the DNA incubated with sample and without Cu (II) (b) was not much cleaved into open circular form and not different with control, without sample (a). However, when Cu (II) was added, almost DNA was cleaved to form open circular form.

Discussion

Green tea is thought as powerful antioxidant. However, strong antioxidant can do dual roles depends on the situation. If H_2O_2 and EDTA-chelated $FeCl_3$ are incubated with ascorbic acid, ascorbic acid reduces the Fe^{3+} into Fe^{2+}, and promotes the Fenton-type hydroxyl radical production. In our experiment, when there are only H_2O_2 and $FeCl_3$-EDTA complex, they could not breakdown the deoxyribose. But green tea extract acted like ascorbic acid, a reducing agent, so it could make deoxyribose into sugar radicals that can fragment into various products. If it occurs in intact DNA, deoxyribose radical can attack the DNA bases and make abasic site, so it can be related into strand breaks.

Living bacterial cells produces O_2^- or H_2O_2 during their basal metabolism, and they have metal ions like Cu^{2+} very close to DNA. In our second experiment, the reaction temperature was 4°C, so bacterial repair systems might not be operated. In that situation, EGT promoted the hydroxyl radical production and produced radical attacked purine, pyrimidine bases. 8-oxoguanine which is the most abundant oxidized base in oxidative stress situation was measured, and it presented the green tea extract's oxidizing capacity compared with normal condition. However, green tea extract couldn't induce dose-dependent mutation in *Salmonella typhimurium* TA 102, which explained that A:T base pair oxidation contained in the mutation mechanism of this strain is not associated with the action of ethanol extract of green tea.

At last experiment, green tea extract cleaved single strand of isolated, supercoiled DNA notably only in the presence of Cu (II) ion. This result confirmed the need of metal ion for the action of oxidative damage of this extract.

Green tea components like catechins and other phenolic compounds have been studied on their anti-oxidative capacity in oxidative stress condition or lipid peroxidation system. However, as shown in this study, ethanol extractable compounds of green tea e.g. catechins, can also promote hydroxyl radical production with the help of Cu ion near the DNA, oxidize the DNA bases, breakdown the deoxyribose and cleave the DNA single strand.

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