

## Chemopreventive Effect of Green Tea (*Camellia sinensis*) among Cigarette Smokers

J. S. Shim, M. H. Kang, Y. H. Kim, J. K. Roh, C. Roberts, and I. P. Lee<sup>1</sup>

Toxicology Research Center, Korea Research Institute of Chemical Technology, Daeduk Danji [J. S. S. Y. H. K., J. K. R.], and Department of Food and Nutrition, Han Nam University [M. H. K.], Dajeon, Republic of Korea; Center for Substance Abuse Treatment, Substance Abuse and Mental Health Service Administration, Rockville, Maryland 20857 [C. R.]; and Molecular Toxicology Branch, Division of Toxicological Research, Food and Drug Administration, Center for Food and Applied Nutrition, Washington, DC 20204 [I. P. L.]

### Abstract

Chemopreventive effects of green tea and coffee among cigarette smokers were examined in 52 clinically healthy male subjects between 20 and 52 years of age. Blood specimens were obtained from nonsmokers (group I), smokers (group II), smokers consuming green tea (group III), and smokers drinking coffee (group IV). The mean number of cigarette smoking years (>10 cigarettes/day) in groups II-IV ranged from 13.4 to 14.7 years. Daily intake of green tea and coffee was 2-3 cups/day for 6 months (groups III and IV). The frequencies of sister-chromatid exchange (SCE) in mitogen-stimulated peripheral lymphocytes from each experimental group were determined and analyzed statistically. SCE rates were elevated significantly in smokers ( $9.46 \pm 0.46$ ) versus nonsmokers ( $7.03 \pm 0.33$ ); however, the frequency of SCE in smokers who consumed green tea ( $7.94 \pm 0.31$ ) was comparable to that of nonsmokers, implying that green tea can block the cigarette-induced increase in SCE frequency. Coffee, in contrast, did not exhibit a significant inhibitory effect on smoking-induced SCE.

### Introduction

Epidemiological data suggest that cigarette smoking is responsible for 85-90% of lung cancers and 30% of all cancers (1). In the United States alone, the number of cigarette smokers is estimated to be 50 million. Lung cancer has been the leading cause of death in men and women, and recently lung cancer mortality in women surpassed that of breast cancer. In spite of well established cancer risks, smokers continue to expose nonsmokers in the workplace and elsewhere. A 30% increase in lung cancer risk is associated with exposure to passive or environmental cigarette smoke (2).

The etiology of cigarette smoke-related cancers is attributed to numerous carcinogens, some of which have been identified, e.g., reactive polycyclic aromatic hydrocarbons, alkylnitrosamines, aromatic amines, aza-arenes, aldehydes,

NNK,<sup>2</sup> and metals (3). A variety of DNA adducts derived either directly or indirectly through activated intermediates has been demonstrated in numerous human tissues, including human lymphocytes (4). The level of DNA adducts correlates directly with tumor formation in some tissues, such as mouse skin (5).

These considerations underscore the urgent need to identify chemopreventive agents to reduce cigarette smoke-induced cancer risk. Green tea (*Camellia sinensis*) has been shown to be antimutagenic and anticarcinogenic (6-9). Recent experimental studies have demonstrated that either p.o. or topical application of (-)-epigallocatechin gallate, one of the major polyphenol components of green tea, prevented tumor initiation as well as tumor promotion initiated by a variety of carcinogens (i.e., PAH, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-nitrosodiethylamine, NNK, azoxymethane, radiation, etc.) in experimental animal tumor models (10-15). In addition, epidemiological studies demonstrated that the death rate of all types of cancer, including stomach cancer in the midwest areas of Shizoka Prefecture (Japan), where green tea is consumed daily, was significantly lower than the national average in Japan (16). A case-control study in Kyushu, Japan, also showed that individuals consuming green tea more frequently or in larger quantities tended to have a lower risk for gastric cancer (17).

Despite a higher average consumption of cigarettes among Japanese males as compared to their United States counterparts, lung cancer mortality in Japan is significantly lower (18). This difference may be attributed to dietary habits and/or genetic factors. The Japanese diet contains far less fat than that of the United States, as well as foodstuffs rich in phytoantioxidants (e.g., soy, green tea, and vegetables). Given the paucity of human studies in the literature, we sought to evaluate the chemopreventive effects of daily green tea consumption in human smokers using sister chromatid exchange frequencies in peripheral lymphocytes as a mutagenic marker.

### Materials and Methods

**Selection of Participants.** Questionnaires were sent to 400 male workers, 20-52 years of age, at the main offices of the Shinung Research Unit and Dajeon Factory, Tae Pyong Yang Cosmetic Company (Seoul and Dajeon, Republic of Korea). The questionnaire design was adapted primarily from Carrano and Natarajan (19). Three hundred fifty-seven completed responses were obtained. Four general selection criteria were then applied: (a) no genetic or other preexisting disease; (b) no known exposure to toxic chemicals or radiation or alcohol; (c) <55 years of age; and (d) no history of serious illness since birth. The resultant 52 selected male subjects were tested with

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<sup>1</sup> To whom requests for reprints should be addressed, at Food and Drug Administration, DTR, HFS 509, 8301 Muirkirk Road, Laurel, MD 20708.

<sup>2</sup> The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; SCE, sister chromatid exchange; HBsAg, hepatitis B virus surface antigen; *F*, the distribution of the ratio of two variances.

respect to hematological and urinalysis parameters and serum chemistry and were evaluated clinically to be healthy.

**Grouping of the Selected Subjects.** Selected subjects were grouped as follows: group I, nonsmokers who were not coffee or green tea drinkers; group II, smokers with no green tea or coffee intake; group III, smokers who drank green tea (2–3 cups/day for 6 months) but no coffee; and group IV, smokers who drank coffee (>2–3 cups/day for 6 months) but no green tea. The mean years of smoking in groups II–IV were  $14.71 \pm 2.18$ ,  $13.50 \pm 2.19$ , and  $13.36 \pm 1.74$ , respectively (Table 2).

**Blood Sample Collection, Culture, and Chromosome Spreads.** Subjects were fasted 12 h prior to phlebotomy. Blood was drawn into heparinized syringes (50 IU/ml sodium heparin). A 25- $\mu$ l plasma aliquot was tested for HBsAg via an HBsAg test kit (Jeil Sugar Co., Seoul, Korea) prior to cell culture. HBsAg-negative blood (0.8 ml) was inoculated in 9.5-ml Eagle's MEM (Flow Laboratory, McLean, VA), supplemented with 100 units/ml of penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO) and heat-treated FCS. Phytohemagglutinin (0.1 ml) and 5 mM 5-bromodeoxyuridine (0.05 ml to a final concentration of 25  $\mu$ M) were added to culture vessels that were incubated at 37°C in 5% CO<sub>2</sub>/95% air for 70 h. Added to this was 0.05 ml of 10  $\mu$ g/ml colchicine (BDH Chemicals, Ltd., Poole, England); after 2-h incubation, cells were centrifuged, resuspended in prewarmed hypo-osmolar solution (150 mOsm KCl) at 37°C, and fixed immediately in repeated changes of 3:1 methanol:acetic acid. Chromosome spreads were prepared by dropping cell samples from 20 cm above glass slides, which were dried on a warmer at 30°C.

**Chromosome Staining.** Chromosomes were stained with the use of a modified fluorescence-Giemsa technique. Slides were placed in 5  $\mu$ g/ml bisbenzimidazole (Sigma) for 10 min and then covered completely with a thin film of PBS. The submerged slides were irradiated under a 2  $\times$  15 W photoactivator lamp at a distance of 10–15 cm for 10 min. Slide preparations were mounted in DePeX (Fluka 44581, Buchs, Switzerland).

**SCE Scoring.** Twenty-five cells were scored per culture. Only diploid second metaphase (M<sub>2</sub>) cells with 45–47 centromeres were scored. Every point of exchange was counted as a SCE. Exchanges at the centromere were included only when twisting at this point could be ruled out.

**Statistical Analysis.** All data were processed with the use of the PC-SAS<sup>+</sup> statistical software program. The Student's *t* test, following Bartlett's test and one-way ANOVA, was applied. The relationships among the categories were tested by Pearson correlation.

## Results

The 52 healthy male subjects chosen for this study were categorized into four groups: nonsmokers (group I), smokers (group II), smokers with green tea intake (group III), or smokers with coffee intake (group IV). Observed levels of SCE in the study subjects were correlated first with 12 serum biochemical variables (RBC count, albumin, aspartate transaminase, alanine transaminase, alkaline phosphatase, glutamate-pyruvate transaminase, blood urea nitrogen, creatinine, cholesterol, high density lipoprotein cholesterol, and glucose), 11 food frequency categories (bean products, meat and fish, eggs, milk products, dried small fish and seaweed, green and yellow vegetables, other vegetables, fruits, fats and fried food, instant foods, and a total food practice score), and 13 demographic factors (Table 1). Correlations among SCE frequencies and biochemical variables, food frequency categories, and other demographic fac-

Table 1 Effect of various factors on SCE frequency of the study subjects

Variable	+/- <sup>a</sup>	No.	SCE frequencies	T value	Probability
Marital status	+	42	8.52 $\pm$ 0.24 <sup>b</sup>	-0.11	0.913
	-	10	8.59 $\pm$ 0.56		
Use of computer	+	10	8.40 $\pm$ 0.64	0.24	0.811
	-	42	8.56 $\pm$ 0.23		
Exposure to chemicals	+	7	7.57 $\pm$ 0.52	1.93	0.086
	-	45	8.68 $\pm$ 0.24		
Smoking	+	43	8.84 $\pm$ 0.23	4.47	0.000 <sup>c</sup>
	-	9	7.03 $\pm$ 0.33		
Intake of vitamin pills	+	5	8.65 $\pm$ 0.72	0.18	0.862
	-	46	8.51 $\pm$ 0.24		
Chronic medication use	+	4	9.40 $\pm$ 0.62	1.39	0.236
	-	46	8.47 $\pm$ 0.24		
Vaccine	+	35	8.52 $\pm$ 0.28	0.06	0.956
	-	17	8.55 $\pm$ 0.37		
Surgery	+	3	7.28 $\pm$ 1.21	1.07	0.390
	-	49	8.61 $\pm$ 0.22		
Intake of processed food	+	14	8.14 $\pm$ 0.40	1.12	0.273
	-	38	8.67 $\pm$ 0.26		
Intake of artificial sweeteners	+	4	7.61 $\pm$ 1.20	0.82	0.469
	-	48	8.61 $\pm$ 0.22		
Cancer patient in family	+	4	8.74 $\pm$ 0.73	0.30	0.784
	-	48	8.51 $\pm$ 0.23		
Coffee intake	+	13	9.23 $\pm$ 0.35	2.14	0.042 <sup>d</sup>
	-	39	8.30 $\pm$ 0.26		
Green tea intake	+	15	7.94 $\pm$ 0.31	1.98	0.055 <sup>e</sup>
	-	36	8.77 $\pm$ 0.28		

<sup>a</sup> +, positive; -, negative.

<sup>b</sup> Mean  $\pm$  SE.

<sup>c</sup>  $P \leq 0.001$  by Student *t* test.

<sup>d</sup>  $P \leq 0.05$  by Student *t* test.

<sup>e</sup>  $P \leq 0.1$  by Student *t* test.

tors were not significant (two-tailed) at the 5% level (Table 1 and data not shown). SCE frequencies of subjects sampled at two different geographical locations with differing occupational status were also not significantly different (data not shown).

The age distribution of the 52 study subjects was categorized by cigarette smoking and green tea or coffee consumption. The average age of subjects in this study was  $34.48 \pm 0.95$  years; the average ages of subjects in group I (nonsmokers), group II (smokers), group III (smoker plus green tea), and group IV (smokers plus coffee) were  $31.33 \pm 1.18$ ,  $35.86 \pm 1.94$ ,  $36.20 \pm 2.03$ , and  $33.29 \pm 1.73$  years, respectively. Group I subjects were younger and had less variability in age than the other groups. A Bartlett's test and 9 one-way ANOVA comparing ages by groups were performed. An *F* test comparing the age variance in groups I and IV was significant ( $P < 0.05$ ); a comparison of mean age in groups I and III was also significant by *z* test ( $P < 0.05$ ). The mean years of smoking in groups II–IV were not different statistically (Table 2).

Mean SCE frequencies in Groups I–IV were 7.03, 9.46, 7.94, and 9.20, respectively (Table 2). Since group I subjects were younger and had less variability in age than other groups, a Bartlett's test and a one-way ANOVA were used for statistical analysis. In the present study, 78% of the selected human subjects were less than age 40 years, and there was no statistical evidence for age-related increase in SCE frequency. The vari-

Table 2 SCE frequency by groups (categorized by smoking, green tea, and coffee intake)<sup>a</sup>

Group	No.	SCE (mean ± SE)	Age (mean ± SE)	Years of smoking (mean ± SE)
I	9	7.03 ± 0.33 <sup>b</sup>	31.33 ± 1.18	
II	14	9.46 ± 0.46 <sup>c</sup>	35.86 ± 1.94	14.71 ± 2.18
III	15	7.94 ± 0.31 <sup>d</sup>	36.20 ± 2.03	13.50 ± 2.19
IV	14	9.20 ± 0.32 <sup>e</sup>	33.29 ± 1.73	13.36 ± 1.74
Total	52	8.53 ± 0.95	34.48 ± 0.95	13.86 ± 1.16

<sup>a</sup> All statistical analyses, see "Materials and Methods."

<sup>b</sup> The comparison of group I versus groups II ( $F = 16.91$ ;  $P = 0.0002$ ) and IV ( $F = 14.17$ ;  $P = 0.0005$ ) was highly significant.

<sup>c</sup> Group II versus group III ( $F = 8.53$ ;  $P = 0.005$ ) was highly significant.

<sup>d</sup> Group I versus group III ( $F = 2.54$ ;  $P = 0.12$ ) and group II versus group IV ( $F = 0.15$ ;  $P = 0.70$ ) were not significant.

ance of SCE means for the 4 groups was not different significantly by Bartlett's test ( $\chi^2 = 3.94$ , degrees of freedom, 3;  $P = 0.27$ ). The differences in SCE frequency among groups I–IV also could not be attributed to duration of smoking (Table 2).

Mean SCE frequencies of the four groups were significantly different when compared by one-way ANOVA in all but two comparisons. The paired comparisons of group I (nonsmokers) versus groups II (smokers;  $F = 16.91$ ;  $P = 0.0002$ ) and IV (smokers plus coffee;  $F = 14.17$ ;  $P = 0.0005$ ) were statistically significant. Notably, the mean SCE frequency of group II (smokers) was significantly different from that of group III (smokers plus green tea;  $F = 8.53$ ;  $P = 0.005$ ). However, a paired comparison of group I (nonsmokers) versus III (smokers plus green tea) was not significant ( $F = 2.54$ ;  $P = 0.12$ ), implying that green tea had blocked the smoking-induced increase in SCE frequency. Coffee had no statistically significant effect on smoking-induced SCE groups (group II versus group IV:  $F = 0.15$ ;  $P = 0.70$ ). A paired comparison of group III (smokers plus green tea) versus group IV (smokers plus coffee) was significant ( $F = 6.35$ ;  $P = 0.015$ ).

In order to separate the effects of smoking cigarettes and consuming green tea and coffee, a linear regression model was applied where SCE was predicted by yes = 1 and no = 0 to each of the 3 variables. The results of these analyses and paired comparisons showed that smoking cigarettes and drinking green tea but not coffee affected SCE frequency significantly and explained 32.7% of SCE variation [ $P < 0.0003$ ; parameters: SCE = 7.03 + 2.6 ( $P < 0.0002$ , smoking group), -1.46 ( $P < .0053$ , green tea group), -0.2 ( $P < 0.7$ , coffee group)]. Equivalently, SCE had a multiple correlation with smoking and consumption of green tea and coffee of 0.572, a high value for biological experiments.

## Discussion

In this study we set out to determine whether green tea (*Camellia sinensis*), rich in polyphenols, or coffee could reduce SCE frequencies in peripheral lymphocytes of cigarette smokers. This assay was ideal given that peripheral lymphocytes are easily accessible and that SCE is a much more sensitive mutagenic biomarker than chromosomal aberrations (20). The present study clearly demonstrates that cigarette smoking significantly increased SCE frequencies in peripheral lymphocytes. The mean SCE frequency for smokers (9.46) was 35% higher than that of nonsmokers (7.03; Table 2). These values are similar to those reported previously (20–28). SCE frequen-

cies have also been shown to depend on dose and duration of smoking (20, 24, 28, 29).

The increase in SCE in smokers likely reflects smoking-induced DNA damage rather than changes in lymphocyte subpopulations (30). This is supported by the presence of exceptionally high SCE frequencies in both peripheral lymphocytes of human smokers and in bone marrow cells of mice exposed *in vivo* to cigarette smoke (22, 31, 32). Furthermore, the peripheral lymphocytes of heavy smokers (40–60 cigarettes/day for 9–58 years) as compared to nonsmokers exhibit a 4–6-fold increase in exchange-type chromosomal aberrations (33, 34). In addition, there are significant correlations between 4-aminobiphenyl-hemoglobin and both cotinine and SCEs, as well as a positive, highly significant correlation between 4-aminobiphenyl-hemoglobin and DNA adduct levels in smokers but not in nonsmokers (35, 36).

In the present study, both the mean and the SEM of SCE frequencies in smokers who drank coffee was lower than in smokers only. Although this tendency was not statistically significant, it has been reported in several earlier studies, wherein caffeine treatment lowered SCE induced by mutagens or carcinogens in both hamster and human lymphocytes (37–39). Caffeine application to skin has also been shown to inhibit both UV-induced mouse skin tumorigenesis and breast tumorigenesis in rats (40–42). A greater number of human subjects in the smoker plus coffee category are needed to clarify the effects of coffee consumption.

It is notable that the present study demonstrated no significant difference in SCE rates between nonsmokers and smokers who consumed green tea regularly (2–3 cups/day), and a significant difference between smokers (group II) and smokers who drank green tea (group III). Thus, to the best of our ability to exclude other confounding factors, green tea appears to block the smoking-induced increase in SCE. Because green tea also contains caffeine in addition to a variety of catechins, some of its protective effect against cigarette smoke-induced SCE may be attributed to an additive and/or synergistic contribution of caffeine. However, the tendency of coffee in our study (smokers plus coffee; group IV) to decrease SCE as compared to smokers only (group II) was small and not statistically significant.

Green tea (*Camellia sinensis*) has been shown to be antimutagenic and anticarcinogenic in experimental animals. These studies demonstrated that either *p.o.* or topical administration of green tea or its major chemical constituent, epigallocatechin gallate, prevented tumor initiation and promotion (9–18). In human subjects, tea consumption has been shown to decrease micronucleus formation induced by smoking (43). HPLC analysis of green tea has shown it to be composed of several polyphenols (as much as 30% by dry weight), most of which are catechins [epigallocatechin gallate (15.1%), epigallocatechin (6.9%), epicatechin gallate (3.0%), epicatechin (1.8%), and caffeine (8.1%)] (13, 44).

The potent chemopreventive mechanism(s) of green tea and its polyphenol constituents remain to be defined. The catechins are known free radical scavengers; gallicolcatechins and the catechin gallates exhibit the strongest antioxidant properties (45). Furthermore, all catechins significantly inhibit cytochrome P-450-dependent monooxygenase(s). On the basis of the structure-activity relationship between epicatechins, epigallocatechin gallate is the most potent inhibitor, suggesting that the galloyl or hydroxyl groups may bind to a cytochrome P-450 catalytic site and interfere with the activation of precarcinogens (46). In the NNK-A/J mouse lung tumor bioassay, both green tea and epigallocatechin gallate, which are known to reduce

tumor multiplicity, inhibited NNK oxidation and NNK-induced DNA methylation when added to incubation mixtures containing lung microsomes (47). However, administration of green tea to A/J mice did not inhibit lung DNA methylation *in vivo* (47, 48). It is intriguing, however, that treatment of A/J mice with green tea or epigallocatechin gallate suppressed NNK-induced formation of 8-hydroxydeoxyguanosine, a common free radical-induced DNA lesion (48).

The etiology of cigarette smoke-related cancer is attributed to numerous procarcinogens and carcinogens, some of which have been identified, *e.g.*, polycyclic aromatic hydrocarbons, NNK, and other nitrosamines, aldehydes, and metals (3). In addition, cigarette smoke contains many oxidants, prooxidants, and free radicals that are known to induce oxidative damage or lipid peroxidation *in vitro*, but their role *in vivo* has yet to be defined clearly (49). We propose that the chemopreventive mechanism(s) of green tea against cigarette smoke-induced SCE occurs by: (a) interaction of polyphenolic catechins with cytochrome P-450 monooxygenase(s) to significantly reduce metabolic activation of carcinogen(s); and (b) scavenging of reactive carcinogenic metabolites by catechins to prevent their molecular initiation at critical target sites. While other mechanisms cannot be excluded at this time, the data presented in this study, as well as in work cited previously, suggest that polyphenolic catechins in dietary foodstuffs may provide clinically significant protection against environmental carcinogens. Pharmacological and toxicological studies are needed to further confirm the efficacy and safety of catechins as chemopreventive agents against human cancer.

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