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RESEARCH PAPER

Effect of Dietary Polyphenon E and EGCG on Lung Tumorigenesis in A/J Mice

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ABSTRACT

Purpose To compare the chemopreventive efficacy of Polyphenon E (Poly E), (−)-epigallocatechin-3-gallate (EGCG) and Polyphenon E without EGCG (Poly E-EGCG) on the development of benzo(a)pyrene (B(*a*)P)-induced lung tumors in A/J mice.

Methods Female A/J mice were given a single intraperitoneal injection of B(*a*)P (100 mg/kg body weight). One week after B(*a*)P injection, animals received AIN-76A purified powder diet containing 0.975% (wt/wt) EGCG, 0.525% (wt/wt) Poly E-EGCG or 1.5% (wt/wt) Poly E for 24 weeks or control diet with no additives.

Results Poly E treatment significantly decreased tumor multiplicity by 52% and tumor load by 64%, while EGCG and Poly E-EGCG did not significantly inhibit lung tumor multiplicity. EGCG was more stable in a complex mixture (Poly E) than as a pure compound.

Conclusion EGCG was ineffective when administered by diet likely due to its instability. Thus, EGCG's efficacy on mice lung tumorigenesis requires the presence of other tea catechins.

KEY WORDS chemoprevention · degradation · EGCG · lung tumorigenesis · polyphenon E

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INTRODUCTION

Polyphenon E (Poly E) is a well-defined, pharmaceutical-grade mixture of green tea polyphenols that contains epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechin gallate (GCG), and (−)-epigallocatechin-3-gallate (EGCG). Poly E has various biological activities, including antioxidation, modulation of enzyme systems for metabolizing chemical carcinogens and inhibition of tumor promotion (1). Poly E administered in drinking water, in diet or by aerosol can inhibit carcinogen-induced lung tumorigenesis (Table I). Poly E administered by aerosol or diet significantly inhibited benzo(a)pyrene (B(*a*)P)-induced lung tumorigenesis in A/J mice (2–5). Poly E in drinking water also inhibited the progression of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumor progression in A/J mice (6). The National Cancer Institute has assisted the development of Poly E as a candidate for human chemoprevention studies.

EGCG is the most abundant catechin found in green tea and comprises 65% of Poly E (Supplementary Material, Table 1). EGCG inhibits cell proliferation, induces apoptosis, induces cell cycle arrest, stimulates angiogenesis, and affects cell signaling pathways (7–10). Pure EGCG inhibited NNK-induced lung tumorigenesis in A/J mice when given

Table I Chemoprevention of Lung Tumorigenesis by Poly E and EGCG

Reagent	Carcinogen	Animal model	Mode of treatment	Results	Ref.
EGCG	NNK	A/J mice	Drinking	Decreased tumor multiplicity	(11)
EGCG	Cisplatin or NNK	A/J mice	Drinking	Decreased tumor multiplicity	(12)
EGCG	Tobacco smoke or NNK	A/J mice	Aerosol	No effect	(13)
Poly E	B(*a*)P	A/J mice	Diet	Decreased tumor multiplicity and load	(4)
Poly E	B(*a*)P	A/J mice	Diet	Decreased tumor multiplicity and load	(5)
Poly E, EGCG	B(*a*)P	A/J mice	Aerosol	Poly E decreased tumor multiplicity and load, EGCG no effect	(4)
Poly E-EGCG, Poly E	B(*a*)P	A/J mice	Aerosol	Poly E decreased tumor multiplicity and load, Poly E-EGCG no effect	(2)
Poly E	NNK	A/J mice	drinking	Decreased tumor multiplicity	(6)

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

in drinking water (11,12). EGCG in the drinking water also reduced the number of cisplatin-induced lung tumors in A/J mice (12). In contrast, aerosol EGCG did not decrease tumor multiplicities or tumor load in B(*a*)P-, NNK- or tobacco smoke-induced lung tumorigenesis (4,13). No studies on the effect of EGCG alone administrated by diet have been reported.

The present study was designed to compare the chemopreventive activities of EGCG, Poly E and Polyphenon E without EGCG (Poly E-EGCG) when administered by diet and to determine to what extent EGCG is responsible for the chemopreventive efficacy of Poly E.

MATERIALS AND METHODS

Reagents

Benzo(a)pyrene (B(*a*)P, 99% pure) and tricaprylin were purchased from Sigma Chemical Co. (St. Louis, MO). B(*a*)P was dissolved in tricaprylin immediately before use in animal bioassays. EGCG, Poly E-EGCG and Poly E were obtained from Tokyo Food Techno Co., Ltd. (Tokyo, Japan). The main difference between Poly E and Poly E-EGCG was the content of EGCG. Lot-specific analysis demonstrated 65% EGCG content in Poly E and 1.4% in Poly E-EGCG (Supplementary Table 1).

Animals and *In Vivo* Treatment

Female A/J mice at 6 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed with wood-chip bedding in an environmentally controlled, clean-air room with a 12-hour light–dark cycle and a relative humidity of 50%. Drinking water and diet were supplied *ad libitum*. The study was approved by the Washington University's Institutional Animal Care and Use Committee. Female A/J mice were given a single intraperitoneal injection of B(*a*)P (100 mg/kg body weight) in 0.2 ml of tricaprylin. One week after B(*a*)P injection, the mice were randomly divided into four groups with ten mice per group. Mice were fed AIN-76A purified powder diet (Dyets, Inc., Bethlehem, PA) containing 0.975% (wt/wt) EGCG, 0.525% (wt/wt) Poly E-EGCG or 1.5% (wt/wt) Poly E. Diets were supplemented with 3% sucrose as previously described (3). Treatments continued for 24 weeks (Fig. 1). Diets were prepared weekly, and fresh diet in the cages changed daily. Foods were prepared with a KitchenAid (St. Joseph, MI) mixer, mixing for at least 1 h. The body weights of mice were measured weekly for the duration of the study. The mice were also monitored daily during the study duration. We did not observe any sign of toxicity or loss of body weight. Mice were sacrificed 25 weeks after exposure to B(*a*)P.

Plasma and Tissue Collection

Mice were euthanized by CO_2. Blood samples from the retroorbital plexus of each animal were collected in EDTA-treated tubes. The blood was centrifuged at 950 g for 10 min at 4°C. The obtained plasma was kept at −80°C for further analysis. Lungs from each mouse were fixed in Tellyesniczky's solution overnight (14) and then stored in 70% EtOH. Lungs were evaluated under a dissecting microscope to obtain surface tumor counts and individual tumor size. Tumor volume was calculated based on the following formula: $V = 4\pi r^3/3$ (15). The total tumor volume in each mouse was calculated as the sum of all tumors. Tumor load was determined by averaging the total tumor volume per mouse in each group.

Determination of EGCG in Diet or Plasma by HPLC

0.2 g EGCG, Poly E-EGCG or Poly E diet, prepared fresh or collected from the top of the feeder jar, after being

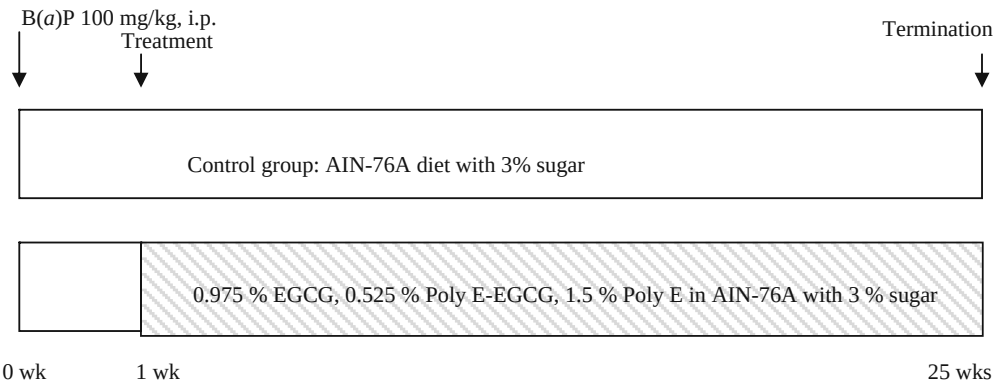


Fig. 1 Protocol for Poly E, EGCG and Poly E-EGCG treatments in B(*a*)P-induced lung tumorigenesis in A/J mice. Mice were given a single i.p. injection of B(*a*)P at 100 mg/kg body weight at 6 weeks of age. Chemopreventive treatments (EGCG, Poly E-EGCG and Poly E) started 1 week after initiation with B(*a*)P. All groups were treated for 24 weeks and terminated 25 weeks after B(*a*)P injection.

placed with the mice in the cage for 1 day (old), were dissolved in 20 ml water, and filtered with a 0.22 μm steriflip Millipore filter (Millipore Corporation, Billerica, MA). The concentration of each catechin was determined by HPLC. The HPLC 1100 series system, consisting of an autosampler, binary pump, temperature-controlled column compartment, and diode array or variable wavelength detector was used (Agilent Tech, Santa Clara, CA). For diet analysis, a 4.6×75 mm Zorbax SB-C18 3.5 μm column was used with a detection wavelength of 244 nm. The mobile phase consisted of ammonium acetate:acetonitrile (80:20), the column temperature was 40°C, and the flow rate of the mobile phase was 1 ml/min. For plasma analysis, a Synergi 4μ Max-RP (Phenomenax, Torrance, CA) column was equilibrated with Buffer A (0.05 M sodium phosphate, pH 7.2 in 25% methanol). After injection, Buffer B (methanol:water:tetrahydrofuran = 70:30:3) was increased from 0 to 8% by a linear gradient between 0 and 3 min, maintained at 8% between 3 and 18 min, and at 55% between 18 and 35 min. The flow rate was 0.8 ml/min. Quantification of EGCG and other catechins was based on calibration curves derived from 5 standards (EGCG, C, EGC, ECG and EC) ranging from 1.8 μg/ml to 1,000 μg/ml.

Statistical Analysis

One tailed Student's *t* test was used to test the *a priori* hypothesis that mean tumor multiplicity and tumor load were decreased by chemopreventive treatments. Data is presented as mean ± standard deviation.

RESULTS

Poly E, but not Poly E-EGCG or EGCG, Decreased Tumor Multiplicity and Tumor Load

Lung tumor incidence was 100% in all groups. B(*a*)P induced 22.6±5.0 tumors per mouse in the untreated control group, and tumor load was 25.9±5.3 mm^3. Mice treated with Poly E diet using a progression protocol (Fig. 1) showed a significant decrease in both tumor multiplicity (52.2% inhibition, 10.8±4.2) and tumor load (64.0% inhibition, 9.3±5.2 mm^3) when compared to the control group. Poly E-EGCG decreased tumor load (27.0%, 18.9±5.7 mm^3), but not tumor multiplicity (Fig. 2).

The concentration of EGCG in the diet is identical in the EGCG and Poly E groups. The greatest difference between Poly E and Poly E-EGCG was the content of EGCG. Poly E contained 65% EGCG, while Poly E-EGCG contained only 1.4%. The difference in tumor multiplicity and tumor load between Poly E group and Poly E-EGCG group was therefore attributed to the presence of EGCG. This implied that EGCG might be the most effective component on chemoprevention. Interestingly, mice fed EGCG alone did not have a reduction in either tumor multiplicity or tumor load.

Stability of EGCG, Poly E-EGCG and Poly E by Diet

The instability of purified EGCG was reported previously (16). In our experiment, we observed an obvious color change in EGCG diet, suggesting oxidation or degradation of EGCG in the diet. We therefore used HPLC analysis to monitor whether EGCG degrades over time. Newly prepared diet (fresh) and diet that had been placed with the mice for 1 day (old), was dissolved in a 25% ethanol solution, filtered and analyzed for catechin content by HPLC. Freshly prepared EGCG and Poly E diets had similar EGCG concentrations (79.9 and 78.4 μg/ml, respectively. Fig. 3). When diets were placed with mice, the concentration of EGCG was decreased to 1.7 μg/ml in the EGCG group and 29.8 μg/ml in the Poly E group. The degradation rate of EGCG was 62.0% in the Poly E diet and 97.7% in EGCG diet. In comparison, the degradation rate of EC in Poly E-EGCG and EGCG diets after placing in experimental housing conditions was only 11.7% and 8.3%, respectively (data not shown). The concentration of EGCG in EGCG and Poly E diets after 10 days of room air

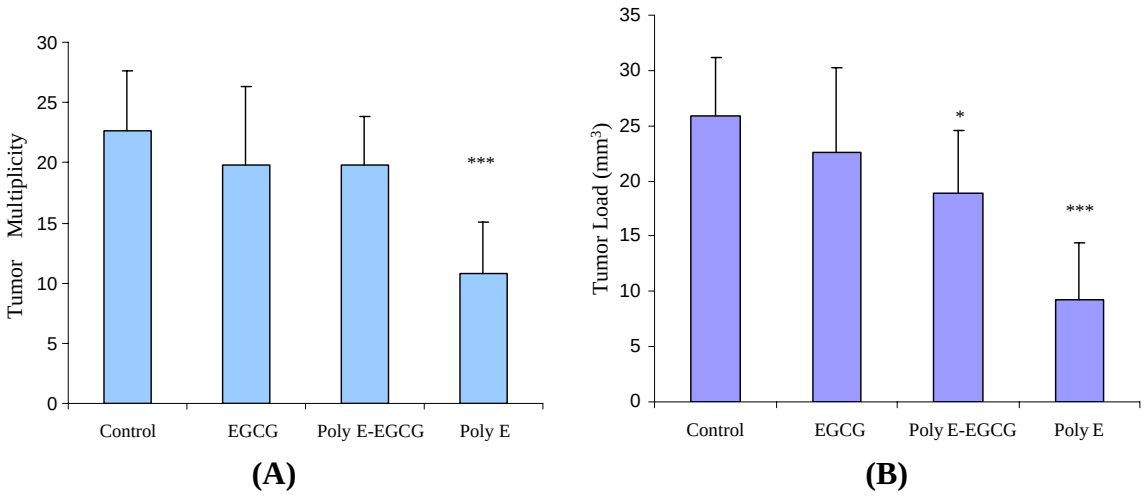


Fig. 2 Effect of green tea polyphenols treatment on B(*a*)P-induced lung tumorigenesis in A/J mice. (**A**) Poly E significantly decreased tumor multiplicity by 52.2%. EGCG and PolyE-EGCG had no effect. (**B**) Poly E decreased tumor load by 64.0%, and Poly E-EGCG decreased tumor load by 27.0%, while EGCG had no effect. *$P < 0.05$, and ***$P < 0.001$, compared with the control group.

exposure was not significantly changed compared to freshly prepared diets (data not shown). This analysis showed that EGCG degrades much faster when placed with the mice than when simply exposed to air. Also, EGCG is more stable when it is present in a complex mixture (i.e. Poly E) than EGCG alone.

Stability of EGCG in Plasma

To determine the effect of diet composition on EGCG bioavailability, we measured the concentration of EGCG in plasma collected from each group. Two peaks (7.2–7.3 and 9.9–10.0 min) other than the EGCG peak (9.1–9.2 min) were present on the HPLC spectra (Fig. 4A) from EGCG-treated mice. A previous study suggested the peak at 9.9 min to be the dimer of EGCG. The identity of peak eluting at 7.2 min was not defined, but was suggested to be a metabolite of EGCG (17,18). In the EGCG-treated mice, the EGCG peak was detectable in only one sample, but the 7.2 min peak was present in all ten samples, and the 9.9 min dimer peak was present in half the samples. In the Poly E group, all ten plasma samples, had the EGCG peak, but only four out of ten samples had the 7.2 min peak, and no dimer peak was found in any sample. The EGCG concentration in plasma was 73.6±232.7 ng/ml in the EGCG group, and 1,065.2±978.2 ng/ml in the poly E group (Fig. 4B). These results suggest that EGCG formed dimers or was degraded when other tea catechins were not present. Hence, EGCG bioavailability decreased in mice treated with EGCG diet alone, suggesting that this might be the reason for the lack of chemopreventive efficacy.

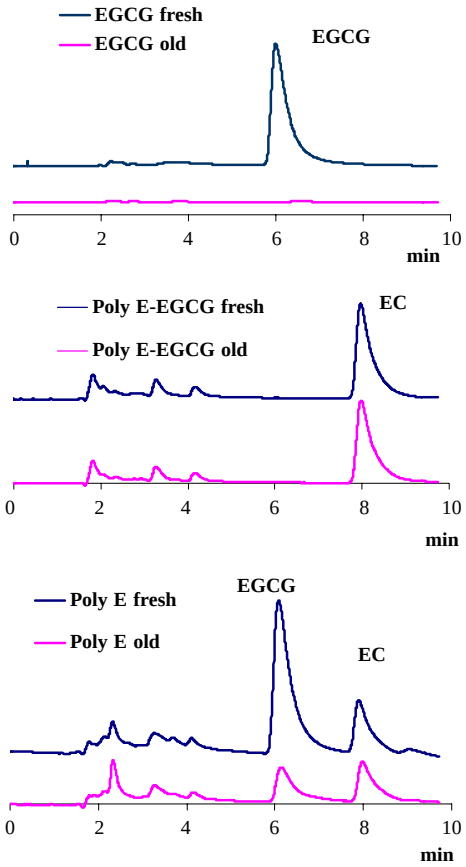
DISCUSSION

Poly E is a well-defined pharmaceutical-grade mixture of tea polyphenols containing different catechins that inhibit the development of lung cancer in animal models (12,14,19–21). EGCG is the most abundant catechin found in green tea, and it has received most of the attention because it can inhibit cell proliferation in many cancer cell lines through different mechanisms (7–10).

EGCG can also prevent mouse lung tumorigenesis when administered in drinking water (11,12). However, no study reporting administration of EGCG in the diet has been previously reported. In the present study, we tested the chemopreventive efficacy of dietary EGCG on B(*a*)P-induced lung tumorigenesis in A/J mice.

The chemopreventive efficacy of EGCG was compared to Poly E using dose that delivered identical amount of EGCG (Poly E diet *versus* EGCG diet). Poly E decreased tumor multiplicity and tumor load when administered in the diet. Poly E-EGCG decreased tumor load, but did not have a significant effect on tumor multiplicity. The main difference between the chemical composition of Poly E and Poly E-EGCG was the content of EGCG. Poly E contains 65.0% EGCG, while Poly E-EGCG contains only 1.4% EGCG. Although there were differences in the ratios among other tea catechins in Poly E and Poly E-EGCG, the absolute amounts of each catechin given at the doses used were similar. The difference in tumor multiplicities and tumor loads between Poly E group and Poly E-EGCG group was therefore attributed to the presence of EGCG. EGCG by diet would be the most effective compound in

(A)



(B)

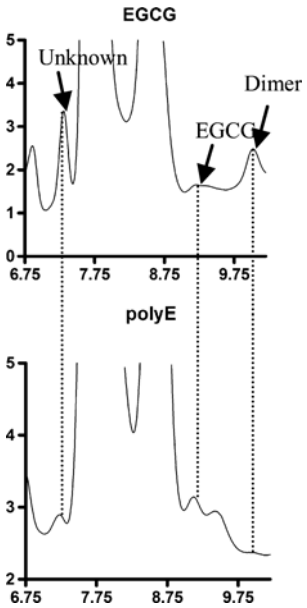
	Concentration of EGCG (μg/ml)	Degradation
EGCG old	1.8	97.7 %
EGCG fresh	79.9	
Poly E old	29.8	62.0 %
Poly E fresh	78.4	

Fig. 3 HPLC quantification of EGCG and EC in diet. (**A**) Typical chromatogram obtained from fresh diet (fresh) and diet placed with mice for 1 day in the cage (old). (**B**) Degradation of EGCG in each diet before and after placing with mice for 1 day in the cage (old). The concentration of each catechin was determined by HPLC and the percent lost calculated.

Poly E chemoprevention based on the results obtained from Poly E and Poly E-EGCG group. Interestingly, treatment with pure EGCG had no effect on tumor multiplicity or tumor load.

EGCG is relatively unstable under neutral or alkaline conditions and could be rapidly degraded by deprotonation of hydroxyl group on the phenol rings. Methylation, glucuronidation and sulfation reduced biological activities of EGCG *in vivo* (22–24). We observed an obvious color change in EGCG diet during the experiment and therefore hypothesized that EGCG may be degraded under the experimental conditions. HPLC analysis indicated that

(A)



(B)

Group	Unknown peak (7.2-7.3 min)	EGCG (9.1-9.2 min)	Dimer EGCG (9.9-10.0 min)
EGCG (ng/ml)	716.9 ± 356.1	73.6 ± 232.7	626.4 ± 659.5
Poly E (ng/ml)	464.5 ± 548.1	1065.2± 978.2	0

Fig. 4 HPLC quantification of EGCG in plasma. (**A**) Typical plasma chromatogram obtained from EGCG- and Poly-E-treated mice. *Arrows* indicate EGCG, dimer and unknown peaks; (**B**) the concentration of EGCG, unknown peak and dimer EGCG in EGCG group or Poly E group, mean ± SD. Blood from the retroorbital plexus of each animal was taken into EDTA-treated tubes. The blood was centrifuged at 950×g for 10 min at 4°C. Ten samples of each group were determined by HPLC.

EGCG is more stable in the Poly E mixture than when present alone in the diet (97.7% degradation in the EGCG diet group *versus* 62.0% in the Poly E group). In both the Poly E and EGCG diets, EGCG was not degraded when stored in room air for 10 days. This observation indicated that the degradation of EGCG is caused by the conditions present in the mouse cages; almost all EGCG was degraded when present alone. It appeared that the degradation by the conditions present in the mouse cages might be the reason that EGCG lost its function on chemoprevention by diet. For the Poly E group, because of the presence of other catechins, the degradation of EGCG was slowed, and may be the reason for improved chemopreventive efficacy.

Difference in degradation was also observed in plasma. The amount of EGCG was lower in plasma collected from the EGCG group compared to the Poly E group. Glucuronidation and sulfation of tea Polyphenols were reported to be the major elimination pathways for EGCG. The competition among tea polyphenols for glucuronosyl-

transferase and sulfotransferase may affect EGCG elimination (16).

Shimizu *et al.* (2005) reported that Poly E may be preferable to EGCG because the mixture of catechins may exert synergistic growth inhibitory effects (25). The presence of other tea catechins might affect the absorption, biologic activity, or other properties of EGCG (20). Because tea catechins have similar chemical structures, metabolism and excretion of EGCG may be reduced and the residence time of EGCG increased when administered in a catechin mixture compared to pure EGCG.

In conclusion, this study confirmed that Poly E was an effective lung cancer chemopreventive agent in female A/J mice when administered in the diet. Pure EGCG administered in the diet was not an effective chemopreventive agent, likely due to preferential degradation of pure EGCG compared to EGCG present in a mixture of catechins. Modifications of EGCG that improve its stability and bioavailability may be an important strategy to improve its pharmaceutical profile.

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