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# Sustained systemic delivery of green tea polyphenols by polymeric implants significantly diminishes benzo[*a*]pyrene-induced DNA adducts

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## Abstract

The polyphenolics in green tea are believed to be the bioactive components. However, poor bioavailability following ingestion limits their efficacy *in vivo*. In this study, polyphenon E (poly E), a standardized green tea extract, was administered by sustained-release polycaprolactone implants (two, 2-cm implants; 20% drug load) grafted subcutaneously or via drinking water (0.8% w/v) to female S/D rats. Animals were treated with continuous low dose of benzo[*a*]pyrene (BP) via subcutaneous polymeric implants (2 cm; 10% load) and euthanized after 1 and 4 weeks. Analysis of lung DNA by $^{32}$P-postlabeling resulted in a statistically significant reduction (50%; p=0.023) of BP-induced DNA adducts in the implant group; however, only a modest (34%) but statistically insignificant reduction occurred in the drinking water group at 1 week. The implant delivery system also showed significant reduction (35%; p=0.044) of the known BP diolepoxide-derived DNA adduct after 4 weeks. Notably, the total dose of poly E administered was >100-fold lower in the implant group than the drinking water group (15.7 versus 1,632 mg, respectively). Analysis of selected phase I, phase II, and nucleotide excision repair enzymes at both mRNA and protein levels showed no significant modulation by poly E, suggesting that the reduction in the BP-induced DNA adducts occurred presumably due to known scavenging of the anti-diolepoxide of BP by the poly E catechins. In conclusion, our study demonstrated that sustained systemic delivery of poly E significantly reduced BP-induced DNA adducts in spite of its poor bioavailability following oral administration.

### Keywords

Green tea polyphenols; Polymeric implants; Plasma levels of EGCG; Benzo[*a*]pyrene; Sustained P450 levels; $^{32}$P-Postlabeling

## Introduction

Lung cancer is the second most common cancer type in men and women in the United States, only less than prostate cancer in men and breast cancer in women. However, lung cancer accounts for the highest cancer-related deaths, 31% in men and 26% in women (1). Effective prevention and treatment strategies are therefore urgently needed.

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Green tea is one of the most popular drinks in the world. It is now drawing more attention because of its possible chemoprotective effects. Green tea polyphenols (GTPs), including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) are believed to be the active components. These bioactive components of green tea along with green tea preparations have been shown to exert their chemopreventive effects *in vitro* (2, 3).

*In vivo*, green tea consumption decreased lung tumor incidence and tumor multiplicity in chemically-induced lung tumor models, including polycyclic aromatic hydrocarbon (PAH)- and nitrosamine-induced tumor models (4–9). However, reviews on epidemiological and clinical studies indicated only a marginal beneficial association between green tea consumption and lung cancer risk (10, 11). Similarly, green tea did not show definite effects on other cancer types, including gastric, colorectal and breast cancer (12–16). One major reason for the lack of consistent protection with GTPs is their low bioavailability following oral intake, which ultimately affects their blood levels (17, 18). Studies have shown that only about 0.1%~3% of EGCG and ECG were bioavailable following oral administration (19–21). In an effort to circumvent the poor bioavailability associated with oral delivery of GTPs as well as many other chemopreventive agents, this laboratory has employed an extended release systemic delivery approach for treatment with potential chemoprotective agents. Using this approach, test agents are embedded in cylindrical polymeric implants, which upon subcutaneously grafting provide continuous systemic delivery of the test agents for an extended duration.

Polyphenon E (poly E), a standardized green tea extract, is well characterized and is frequently used and well-accepted in the scientific literature of peer-reviewed journals as a model representative for concentrated green tea extracts for characterization purpose (8, 22, 23). For example, Poly E has been investigated in clinical trials for prostate cancer (22) and has been approved by the FDA for use to treat genital and perianal warts in ointment form (Veregen®, MediGene AG, Munich, Germany). Poly E is in our opinion, an ideal agent to evaluate improvements in bioavailability and systemic delivery of green tea.

Benzo[*a*]pyrene (BP) represents a model PAH to study lung cancer. It is also one of the most potent and environmental carcinogens present ubiquitously in tobacco smoke, automobile exhaust emissions, grilled foods and other sources (24, 25). In this study, poly E was administered by polymeric implants or via drinking water to female Sprague-Dawley (S/D) rats. Animals were challenged with continuous low-dose BP via subcutaneous polymeric implants. Effects of poly E administered by the two routes were compared to determine its efficacy against DNA adducts induced by continuous exposure to BP. Potential mechanisms of action of poly E were also investigated by analysis of mRNA, protein level and enzymatic activity of several phase I, phase II and nucleotide excision repair enzymes.

## Experimental Procedures

### Chemicals

Poly E was a generous gift from Pharma Foods International Co., LTD (Kyoto, Japan) and, according to the certificate of analysis provided by the manufacturer, contains 99.8% polyphenols, of which, catechins compose 89.1%. Further, we measured the content of EC, ECG, EGC and EGCG specifically by HPLC-UV, which accounted for 81.2% of Poly E used in these studies (shown in Figure 3). Additional catechins, not measured here, may also be present in this mixture but to a much lower extent (eg. Gallocatechin gallate). EC, EGC and ECG were purchased from Sigma-Aldrich (St. Louis, MO). EGCG was from LKT laboratories, Inc. (St. Paul, MN). Chemicals used in $^{32}$P-postlabeling DNA adduct analysis were the same as described previously (26). All other chemical reagents were purchased

from Sigma-Aldrich (St. Louis, MO). *Anti*-CYP1A1, GSTM1, UGT1A primary antibody and anti-rabbit HRP-linked secondary antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). *Anti*-β-actin primary antibody was from Sigma-Aldrich (St. Louis, MO). *Anti*-CYP1B1 primary antibody was obtained from BD Biosciences (San Jose, CA). *Anti*-mouse secondary antibody was from Cell Signaling Technology (Danvers, MA). Other reagents used were from the following sources: Trizol (Invitrogen Corporation, Carlsbad, CA), high capacity reverse transcription kit and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL.), and ECL plus detection kit (Amersham Biosciences, Piscataway, NJ). Caution: BP is mutagenic and carcinogenic. Protective clothing should be worn, and appropriate safety procedures should be followed when working with this compound.

## Polymeric implants

Polymeric implants of poly E and BP were prepared by embedding them in a polymeric matrix comprised of a water-insoluble polymer, polycaprolactone (P65) (mol. wt. 65,000). Briefly, the polymer P65 (8 g) was dissolved in dichloromethane (150 ml) and added to a solution of poly E (2 g) in ethanol (30 ml). For BP implants, the polymer (5.4 g) and BP (0.6 g) were both dissolved in dichloromethane (110 ml). Following evaporation of the solvent(s) at 70°C with agitation with a glass rod, the polymer matrix was completely freed of the solvents under reduced pressure overnight. The polymeric material was then filled in a disposable syringe attached to a silastic tubing (I.D. 3.2 mm), heated at 70°C and extruded. The implants were removed from the silastic tubing mould and excised to desired length. Sham implants were also prepared using dichloromethane essentially as poly E implants.

## *In vitro* release of poly E from the implants

Release of poly E was investigated by stirring the implants (2 cm, 200 mg implant containing 40 mg poly E) in phosphate-buffered-saline (PBS) supplemented with 10% bovine serum, pH 7.4 at 37°C to simulate the in *vivo* environment. The amount of catechins released was measured spectrophotometrically at 540 nm after reaction with a dyeing solution containing 0.1% ferrous sulfate and 0.5% potassium sodium tartrate tetrahydrate (27).

## Animal handling

All animal experiments were performed after obtaining approval from the Institutional Animal Care and Use Committee (IACUC). Six week-old female S/D rats were purchased from Harland-Sprague-Dawley (Indianapolis, IN). After acclimation for 3 d, animals were randomized into six groups and provided 4% Teklad diet. One week later, animals were treated with BP, poly E or sham treatments as follows:

Group 1, No treatment

Group 2, Sham implants

Group 3, BP implant (2 cm, 200 mg implant containing 20 mg BP)

Group 4, BP implant + poly E implants (two, 2-cm 200 mg implant containing a 40 mg poly E/implant)

Group 5, BP implant + poly E in drinking water (0.8% w/v)

Group 6, Poly E implants

BP treatment was provided by subcutaneous polymeric implants in order to provide a continuous low-dose carcinogen exposure instead of a bolus dose as this would represent a more realistic *in vivo* exposure situation. Sham, BP and poly E implants were grafted

subcutaneously under anesthesia as described previously (28). Poly E was given in drinking water two days prior to BP implantation. Poly E solution was prepared in deionized water every other day by heating the solution at 90°C with stirring for 3 min. This solution was cooled and stored at 4°C until use. Animals from Groups 1–6 (n = 5) were euthanized one week following BP implantation (Group 6 had only 2 animals). Additional animals in Groups 1–4 (n = 5) were euthanized 4 weeks following BP implantation. Lung and liver tissues were collected and stored at −80°C until use. Blood was collected by cardiac puncture and plasma was collected by mixing with heparin and centrifugation. One ml plasma sample was mixed with 20 μl of 0.4 M $NaH_2PO4$ containing 20% ascorbic acid and 0.1% EDTA (pH 3.6) and stored at −80°C until analysis as described (29).

## Stability of GTPs in polymeric implants

Implants were dissolved in 10 ml of dichloromethane and ethanol (1:1), followed by extraction with water and centrifugation. The supernatant containing poly E catechins was filtered by passing through a 0.22 μm centrifugal filter (Millipore Corp., Billerica, MA). Finally, the eluate was analyzed by HPLC (Shimadzu Corp., Columbia, MD) coupled with a C18 Sonoma column, 25 cm×4.6 mm, particle size of 5 μm (ES industries, West Berlin, NJ) and detected by a UV detector. Mobile Phase A was water containing 0.05% trifluoroacetic acid (TFA), and Phase B was acetonitrile Containing 0.05% TFA. The column was eluted with a linear gradient from 12% to 21% mobile Phase B in 25 min, and increased to 29% for another 10 min at a flow rate of 1 ml/min. Unused implants and implants recovered from the animals were stored under argon until analysis of poly E catechin levels.

## Measurement of poly E doses

The total doses of poly E administered by the implants or via drinking water were calculated as follows:

$$\text{The dose of poly E in the implant group} = \text{initial amount of poly E per two implants} - \text{residual amounts in two implants}$$

The initial and residual amounts of poly E in the implants were measured by dissolving implants in a dichloromethane:ethanol mixture and extraction of the mixture with water as described above, followed by derivatization of the poly E catechins and spectrophotometric measurements (27).

$$\text{The total dose of poly E in the drinking water group} = \text{the concentration of poly E (0.8\%)} \times \text{the amount of water consumed daily per animal} \times \text{duration of the study in days.}$$

## Isolation of DNA, RNA, and microsomes and cytosolic fractions

**DNA isolation—**DNA from lung tissue was isolated by a solvent extraction procedure involving isolation of crude nuclei, removal of RNA and protein by sequential treatments with RNases and proteinase K, respectively, extractions with phenol, phenol:Sevag and Sevag and finally precipitation of DNA with ethanol (26). DNA concentration was estimated spectrophotometrically.

**RNA isolation—**RNA from lung tissue was isolated using Trizol reagent following the Vendor's protocol. The quantity and purity of RNA was tested by Nanodrop. The integrity of RNA was tested by agrose gel.

**Microsome and cytosolic fractions—**Lung tissue was homogenized in 0.25 M sucrose buffer with 0.1 mM EDTA (pH 7.4), centrifuged at 11,000×g for 20 min, followed by centrifugation of the supernatant at 100,000×g for 60 min. The supernatant was collected as cytosolic fraction. The pellet containing microsomes was resuspended in sucrose buffer. Protein concentrations of microsomal and cytosolic fractions were determined by using a BCA protein assay kit.

## Analysis of DNA adducts

DNA adducts were analyzed by $^{32}$P-postlabeling as described (26). Briefly, 10 μg DNA was digested with a mixture of micrococcal nuclease and spleen phosphodiesterase. Before further treatment with nuclease P1 to enrich adducts, an aliquot was used for evaluation of normal nucleotide levels. Adducts and normal nucleotides were labeled in parallel with [$\gamma^{-32}$P]ATP and T4 polynucleotide kinase. Labeled adducts were separated by multi-directional polyethyleneimine (PEI)-cellulose TLC in the following solvents: D1 = 1.0 M sodium phosphate, pH, 6.0; D3 = 4 M lithium formate/7 M urea, pH 3.5; D4 = 4 M ammonium hydroxide/isopropanol (1.1:1), D5 = 1.7 M sodium phosphate, pH 6.0; D2 development was omitted. Normal nucleotides were resolved in 0.18 M sodium phosphate, pH 6.0 by one-directional PEI-cellulose TLC. Adducts and normal nucleotides were detected and quantified by Packard InstantImager. The adduct levels were calculated as relative adduct labeling (RAL), i.e., RAL = cpm of adduct/cpm of normal nucleotides × 1/dilution factor. The levels were expressed as adducts/$10^9$ nucleotides.

## mRNA expression

mRNA levels in the lung tissues were determined by qPCR. Briefly, cDNA was synthesized by using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA). Polymerase Chain Reaction primers for CYP1A1, CYP1B1, Ephx1, UGT1A1, UGT1A6, SULT1A1, GSTM1, ERCC5, ERCC6, XPC and β-Actin were designed using Primer Express (Version 3.0, Applied Biosystems, Foster City, CA). Primer sequences for 18S RNA was obtained from literature (30). The primer sequences were as follows:

| | Forward primer | Reverse primer | Amp (bp) |
|---|---|---|---|
| CYP1A1 | 5-′TGGAGACCTTCCGACATTCAT-3′ | 5′-GGGATATAGAAGCCATTCAGACTTG-3′ | 88 |
| CYP1B1 | 5′-AACCCAGAGGACTTTGATCCG-3′ | 5′-CGTCGTTTGCCCACTGAAAA-3′ | 101 |
| Ephx1 | 5′-ACTTACACATCCAAGCCACCAA-3′ | 5′-GGCCCACGGGAGAGTCA-3′ | 66 |
| UGT1A1 | 5′-ACACAGATCGCATGAACTTCCTG-3′ | 5′-AGGACTCAGAAGGTCCTTGACAGTC-3′ | 151 |
| UGT1A6 | 5′-AGACCACATGACATTTCCCCAA-3′ | 5′-AGAGTTCTGGTGTAAGGCAGGTAGG-3′ | 151 |
| SULT1A1 | 5′-AGCTGAGACACACTCACCCTGTT-3′ | 5′-ATCCACAGTCTCCTCGGGTAGA-3′ | 122 |
| GSTM1 | 5′-TCTTGACCAGTACCACATTTTTGAG-3′ | 5′-TCGAAAATATAGGTGTTGAGAGGTAGTG-3′ | 143 |
| ERCC5 | 5′-GCCGTGGATATTAGCATTTGG-3′ | 5′-GGAGCATCACCATCAAATACAAAA-3′ | 161 |
| ERCC6 | 5′-CTCCAATGCTTCCCCAGTACA-3′ | 5′-CGGGTTTATCGTGTCTCTCAAGA-3′ | 73 |
| XPC | 5′-GGAGGAGGTGGAAGAACTTACTGA-3′ | 5′-CCGCGAGGTAGCAGAATTTT-3′ | 64 |
| 18S-RNA | 5′-GGGAGGTAGTGACGAAAAATAACAAT-3′ | 5′-TTGCCCTCCAATGGATCCT-3′ | 101 |
| β-Actin | 5′-GCCAACCGTGAAAAGATGAC-3′ | 5′-ACCCTCATAGATGGGCACAG-3′ | 165 |

All primer pairs have been tested for their amplification efficiency. β-Actin and 18S RNA were determined to be good reference genes in this study. Polymerase chain reaction was performed with a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). The comparative CT method was used to determine the difference in mRNA expression between samples by normalizing to housekeeping genes (β-actin and 18S-RNA). The fold differences were calculated as ($2^{-\Delta\Delta Ct}$).

### Western blotting

10% SDS polyacrylamide gel was used for separation of microsomal and cytosolic proteins (12 μg each well). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes. After co-incubation with primary and secondary antibodies, detection of proteins was performed using an ECL plus detection kit (Amersham Biosciences, Piscataway, NJ). Primary antibodies were used after the following dilutions: anti-CYP1A1 (1:2000), CYP1B1 (1:2000), GSTM1 (1:400), UGT1A (1:2000) and anti-β-actin antibody (1:4,000). All secondary antibodies were used at 1:3,000 dilution.

### Enzymatic activities

**CYP1A1 and CYP1B1—**The assay was performed using the conditions described (31, 32). Briefly, the reaction mixture contained 100 mM potassium phosphate, pH 7.6, 5 μM ethoxyresorufin, 25 μg of microsomal protein and 250 μM NADPH. Excitation wavelength of spectrometer was set at 530 nm and emission at 585 nm. Readings were taken immediately after the addition of NADPH and continued for over 30 min. The activity was calculated from the linear portion of the plot.

**GST activity—**The activity was measured spectrophotometrically as described (32, 33). Briefly, the reaction mixture contained 100 mM potassium phosphate, pH 6.5, 1 mM 1-chloro-2,4-dinitrobenzene, 5 μg of cytosolic protein and 5 mM reduced glutathione. Absorbance was measured at 340 nm. Readings were taken immediately after the addition of reduced glutathione and continued for over 40 min. The activity was calculated from the linear portion of the plot.

### Plasma levels of Poly E catechins by LC/MS

Quantification of poly E catechins was performed using LC/MS. Plasma sample preparation followed the literature (29). Briefly, 200 μl plasma was mixed with 10.5 μl of glacial acetic acid and 20 μl of a mixture of β-glucuronidase (2,000 units) and sulfatase (43 units) (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 45 min. The reaction mixture was extracted with dichloromethane followed by ethyl acetate extraction twice. Ten μl of 2.5% ascorbic acid was added to the combined ethyl acetate extracts. The mixture was lyophilized and the residue was dissolved in 40 μl 8% acetonitrile. LC/MS was performed by Accela LC from Thermo Scientific (San Jose, CA) with a Hypersil GOLD 50 × 2.1 mm C18 column. A 15 min gradient with 5% acetonitrile/0.1% formic acid (Solvent A) and 95% acetonitrile/0.1% formic acid (Solvent B) at 0.1 ml/min was used. The gradient started from 5% Solvent B that increased linearly to 50% in 12 min and then increased linearly to 75% in 3 min. Elute from LC was coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) via an ESI source. MS and MS/MS spectra were acquired in positive ion mode at 30,000 mass resolution. Peak areas for quantification were obtained from ion chromatograms of monoisotopic molecular ions drawn with 0.06 Th window. Plasma from untreated animals served as blank. Blank plasma spiked with 1, 2, 5, 10, 25, 50, 100, 200, 400 pmol of each catechin standards (EGC, EC, EGCG and ECG), along with 50 pmol quercetin as an internal standard, were used to generate a calibration curve.

### Tissue levels of GTPs by LC/MS

Lung tissue (200 mg) was mixed with 0.84 ml of PBS and 40 μl of glacial acetic acid and homogenized. After centrifugation (16,000g, 6 min), 0.62 ml of the supernatant was processed essentially as described above for plasma GTP levels.

### Statistical analysis

Results were reported as mean ± SD. Generalized linear model (GLM) was used to investigate the effect of treatment and time. SARS v.9.2 was used for statistical analyses. A p-value less than 0.05 was considered statistically significant.

## Results

### *In vitro* release of poly E from the implants

Agitation of poly E polymeric implants in PBS in the presence of serum showed a continuous release of the poly E catechins from the implants. There was a burst release initially and then it declined slowly but continuously. For example, more than 3.6 mg catechins were released on day 1 before declining to nearly 0.27 mg daily release on day 7, thus an exponential decrease occurred as a function of time ($R^2$>0.99) (Figure 1). The cumulative release after 7, 28 and 56 days were 19%, 26% and 29% of the initial amount, respectively. In a separate study we determined that poly E was unstable in PBS with serum degrading by 58% over 24-h period. Therefore, the above data of catechin release have been corrected by multiplying with the factor 2.38.

### Effect of poly E on BP-induced DNA adduct levels and its rate of release and stability *in vivo*

Treatment of S/D rats with a subcutaneous BP implant produced two major DNA adducts in the lung (Figure 2A–b); no adducts spots were detectable in sham-treated animals (Figure 2A–a). These adducts have been characterized previously as derived by interaction of deoxyguanosine with *anti*-BP-7,8-diol-9,10-epoxide (*anti*-BPDE) (adduct 1) (34) and 9-OH-BP-3,4-epoxide (9-OH-BP) (adduct 2) (35). Both adducts were found to have similar adduct levels in lung tissue following 1 week of BP treatment, 11.8 ± 5.2 adducts/$10^9$ nucleotides for BPDE-derived and 13.8 ± 4.5 adducts/$10^9$ nucleotides for 9-OH-BP-derived adducts. The total adduct levels increased by 20% from 1 week (25.6 ± 3.9 adducts/$10^9$ nucleotides) through 4 weeks (31.1 ± 5.2 adducts/$10^9$ nucleotides) of BP treatment (Figure 2). BPDE-derived adducts increased significantly (p=0.011), while a trend was obvious in 9-OH-BP-derived adducts, indicating that both adducts accumulated with time.

Intervention with Poly E administered by the implant route (12.9 ± 2.3 adducts/$10^9$ nucleotides) resulted in a significant reduction (50% decrease; p=0.023) of total adduct levels after 1 week of BP treatment (Figure 2B1); the levels of both adducts were diminished similarly. The implant route of poly E administration was also effective in reducing the adduct burden after 4 weeks. *Anti*-BPDE-dG adduct levels were significantly reduced (35%) while 9-OH-BP-dG adduct levels were decreased (20%) but the reduction was not statistically significant (Figure 2B2). Poly E administered via the drinking water modestly (34%) diminished the total adduct burden after 1 week, with similar effects on both adduct levels, however, this decline was not significant (p=0.20) (Figure 2B1); the effect of poly E via the drinking water route after 4 weeks was not investigated.

There was no significant difference in either the body weight or the lung and liver weights between any of the groups (data not shown) suggesting that BP or poly E administration, irrespective of the route of administration, had no detectable adverse effects.

To determine if poly E catechins embedded in the polymeric matrix were stable during the course of the treatment, extracts of the implants recovered from the animals were analyzed for their integrity by HPLC. Analysis of the extracts from implants before and after the animal treatment and their comparison with reference catechins showed no qualitative difference in the major catechins detected in the implant extracts (Figure 3), indicating that

the catechins remained stable in the implant during the preparation of implants as well as during the course of the study *in vivo*.

To determine the total dose of poly E administered via the implants, we measured the residual amount of poly E in the implants recovered from the animals. The total dose administered via the drinking water was estimated based on the approximate daily water intake. Comparison of the total dose administered during the course of the 1 week study via the implants (15.7 mg) and the drinking water (1,632 mg) indicated that the oral dose was over 100-fold higher than the implant route. Additionally, the doses of poly E released from implants *in vivo* (15.7 mg) and *in vitro* (15.2 mg) at 1 week time point are in excellent agreement, indicating that the *in vitro* release system can predict the *in vivo* rate of release of poly E catechins.

## Plasma levels of GTPs

The calibration curve generated by spiking plasma from untreated animals with 1, 2, 5, 10, 25, 50, 100, 200, 400 pmol of each reference catechin (EGCG, ECG, EGC, EC) and 50 pmol of quercetin as an internal standard was found to be linear in this range ($R^2 > 0.997$ for all compounds tested).

There were no detectable GTPs in plasma of animals (groups 1, 2 and 3) without any poly E treatment based on the LC/MS analysis of the plasma samples. However, animals treated with poly E implants for 1 week clearly showed the presence of all the four GTPs in the plasma samples (Figure 4), with EGCG predominating (Table 1). In the drinking water group, however, EGC and EC were found at significantly higher levels (Table 1). The plasma levels of EGCG in the implant (60.6 ± 25.4 ng/ml) and drinking water (96.9 ± 43.9 ng/ml) groups were not significantly different. EGCG was also the most prominent GTP detected after 4 weeks of poly E implantation; however, the levels were lower compared with one week treatment (Table 1).

## Tissue levels of GTPs

The detection limits for EGCG, EGC, EC and ECG were established first in the lung tissue environment and found to be approximately 1, 3, 3 and 4 ng/g, respectively. Lung levels of EGCG at 1 week time point were found to be 17.2 ± 8.50 and 19.8 ± 6.87 ng/g tissue in animals treated with poly E via implants (Group 4) and drinking water (Group 5), respectively. However, none of the other catechins EGC, EC and ECG were detected in the lung tissue presumably they were below the detection limits.

## Effect of poly E on xenobiotic-metabolizing and DNA repair enzymes in lung tissue

Compared with sham treatment, BP treatment by low-dose continuous exposure resulted in substantial overexpression of CYP1A1 (192 ± 8.5 fold) and CYP1B1 (15 ± 1.2 fold) after 1 week as determined by qPCR. The effect on CYP1A1 expression after 4 weeks was even more pronounced (852 ± 25.9 fold) though the effect on CYP1B1 expression (8 ± 0.68 fold) was less pronounced (Figure 5). Poly E administration alone or together with BP treatment showed no significant effect on the expression of the selected phase I enzymes (CYP1A1, CYP1B1 and Epxh1), phase II enzymes (UGT1A1, UGT1A6, SULT1A1 and GSTM1) or DNA repair enzymes (XPC, ERCC5 and ERCC6) (Figure 5).

Consistent with the findings at the mRNA level, sham implant treatment did not affect the expression of CYP1A1, CYP1B1, UGT1A and GSTM1 at the protein levels. However, treatment with BP implants showed higher levels of CYP1A1 and CYP1B1 after 1 week of BP treatment consistent with their overexpression at the mRNA levels. These proteins still remained overexpressed following 4 weeks of BP treatment compared with sham treatment,

though the levels were less pronounced; poly E administration alone (group 6) showed no significant effects on any of the phase I and Phase II enzymes studied (Figure 6).

At the activity level also, BP treatment greatly induced the activity of CYP1A1 and CYP1B1 compared with sham treatment in the lung microsomes consistent with their overexpression at the mRNA and protein levels. However, no significant inhibition of the activity was observed by poly E administered by implants together with BP. The enzymatic activity was, in fact, significantly increased ($p=0.035$) by poly E when administered via the drinking water together with BP implant. However, a significant inhibition of enzymatic activity ($p=0.004$) was observed by poly E administered by implant between $4^{th}$ and $1^{st}$ week (Table 2). No significant change in the GST activity was observed following any of the treatment given individually or in combination (Table 2).

## Discussion

GTPs have been shown to have significant chemoprotective activity *in vitro* such as antiproliferative, pro-apoptotic activity, however, their efficacy *in vivo* is inconsistent. A primary limiting factor of this discrepancy is believed to be poor bioavailability of GTPs. In this study we have investigated the application of a sustained-release system via subcutaneous polymeric implants in rats by poly E containing a mixture of GTPs as an alternative to the oral administration of these chemoprotective compounds to increase their bioavailability and efficacy *in vivo. In vitro* release of the GTPs from the poly E implant showed a continuous decline with time. This observation in the release appears to be a simple diffusion process, in which the rate of release was inversely proportional to the square of the distance between the molecules and the implant surface. In this sense, surface-bound drug of the implants is released more readily compared with the drug molecules embedded in the inner layers of the implant. Further, GTPs are not stable under alkaline pH, but stable in acidic enviroments. In the *in vitro* release experiment, degradation of the GTPs occurs as they are released from the implants and enter into in the surrounding medium (PBS with 10% bovine serum). In this study the rate of degradation of the GTPs was determined separately and the rate of release was adjusted using a correction factor. Although degradation of the GTPs occurs following their release from the implant, no degradation was observed during preparation of the implant due to the stability of GTPs in the organic solvents used in the preparation process. Once the GTPs are embedded in the polymeric matrix, they are sheltered from any aerobic oxidation. The stability of Poly E in water over a 24 h period was also tested. Water generally has a pH 6~7. HPLC analysis indicated that Poly E in water is quite stable such that less than a 5% loss of the initial amount of Poly E was observed. Based on this study, we conclude our preparation of Poly E in drinking water for this study provided essentially the targeted dose of Poly E to the test animals.

Previous studies have indicated that green tea and its bioactive components have protective effects against PAH-mediated DNA damage and carcinogenesis, including BP. Preparations of green tea, administered during or after PAH exposure, have been shown to decrease tumor incidence and multiplicity in animal models including mouse forestomach and lung (4), mouse transplacental lung tumors (36) and hamster buccal pouch (23). Inhibition of PAH-mediated DNA damage (37), including our own studies (38) and mutagenesis (39) have been shown to mediate green tea's antitumorigenic and anticarcinogenic effects. In this study, the GTP-containing poly E implants were found to be more effective in reducing BP-induced DNA adducts in rat lung tissue following a 1 week exposure, compared to poly E administered via the drinking water (50% versus 34% reduction). More importantly, we determined that the total dose of poly E administered via the implants was >100 times lower than that administered by the oral route (15.7 mg versus 1,632 mg). This observation

supports our hypothesis that subcutaneous polymeric implants provide an improved delivery system of GTPs over that of oral administration. Bioavailability of EGCG was also found to be improved by transdermal delivery compared to oral dosing (40). Poly E implants also continued to be an effective inhibitor of BP-induced DNA adduct formation following 4 weeks of exposure of the rats to continuous low doses of BP. However, the DNA adduct inhibition activity was reduced compared the 1 week treatment, presumably due to decreased release of the GTPs from the implants; the poly E effect via the drinking water route after 4 weeks was not investigated.

In order to further investigate the mechanism(s) of poly E's observed BP–induced DNA adduct inhibition, analysis of several enzymes involved in PAH metabolic activation (CYP1A1, CYP1B1 and Ephx1) and detoxification (UGT1A1, UGT1A6, SULT1A1, GSTM1) as well as others involved in nucleotide excision repair (ERCC5, ERCC6, XPC) was conducted. Reports of the effect of GTPs on P450 showed mixed results. Some cell culture studies have shown that green tea extracts can act as an agonist of the AhR and induce the expression of CYP1A at the mRNA and protein levels (41, 42), while simultaneously as an antagonist to inhibit CYP1A expression induced by TCDD (41). However, other studies suggested that the effect of GTPs on P450 monooxygenase varies with cell type (43) and tissue type (44). Many confounding factors, including but not limited to, the differences in composition of GTP preparations in various studies, *in vitro* versus *in vivo* administration routes and dosage may contribute to the observed inconsistence. Presumably due to similar reasons, there were conflicting reports of the effect of GTPs effects on phase II enzymes such as GST (23, 45, 46). In this study, continuous exposure to low dose BP via polymeric implants dramatically increased the expression of CYP1A1 and 1B1 at both mRNA and protein levels, while having no effect on GSTM1 expression. In agreement with this, the enzyme activity of CYP1, but not GST was significantly higher following BP implant treatment. Poly E, administered via the drinking water, but not by the implants, was found to further increase CYP1 enzymatic activity induced by BP, which may, in part, account for the decreased efficacy of the oral exposure route as compared to the implant route regarding the inhibition of DNA adduct formation between these two groups. However, with the exception of CYP1 enzymatic activity, poly E, administered either by implant or orally, had no effects on the expression or activity of any of the metabolizing or DNA repair enzymes studied, suggesting that poly E inhibits BP-induced DNA adduct formation through non-enzymatic pathways. One likely pathway for the inhibition of BP-induced DNA damage is direct binding of the electrophillic metabolite of BP with the adjacent hydroxyl groups of the green tea polyphenols. In a recent study published from this laboratory (38), a correlation between the number of adjacent aromatic hydroxyl groups in the structure of various GTPs and hydrolyzable tannins and their potencies for inhibiting BP-induced DNA adduction was found. Further, electospray ionization mass spectrometry and liquid chromatography-mass spectrometry analysis confirmed the direct covalent interaction of the hydroxyl groups of a model GTP, EGCG, with *anti*-BPDE, the ultimate carcinogenic metabolite of BP.

EGCG is the most active and also the most abundant component in poly E. The biological effects in this experiment are hypothesized to be predominantly mediated by EGCG and possibly ECG albeit to a lesser degree. The dramatic difference in plasma levels of EC and EGC in the poly E implant group and drinking water group at the 1 week time point reflects the large difference (>100 times fold) in the total dose administered, as noted previously, while the absorption of EC and EGC from the digestive tract is relatively high (31.2% and 13.7%, respectively as reported) (19). The plasma level of EGCG is comparable between these two groups despite the large dose difference, presumably due to the relatively poor digestive absorption of EGCG (0.1%) (19). Considering the short half lives (<3–4 h) of these catechins *in vivo* (19), the plasma level of the catechins primarily reflects the poly E

released into blood stream by the implant or absorbed from the digestive tract on the last day or even a shorter time duration.

Separate experiments indicated that the Poly E release profile from the implants *in vitro* mimic the *in vivo* release pattern (data not shown). In this experiment, the doses of poly E released from implants *in vivo* and *in vitro* after 1 week are also in excellent agreement, indicating that the *in vitro* release system can predict the *in vivo* rate of release of poly E catechins, *In vitro* release data showed the release at day 1 is more than 13–fold higher than the release at day 7. So, it is reasonable to speculate that the plasma concentration of GTPs in the implant group may be much higher initially. In the drinking water group, the plasma concentration of GTPs somewhat fluctuated based on the water intake. The significant reduction in the DNA adduct levels observed after 7 d by poly E implants seem to result from its cumulative release during the 7 day period, not from just the $7^{th}$ day. This notion may explain the higher degree of adduct inhibition observed in the implant versus the drinking water groups.

In conclusion, our study demonstrate that sustained systemic delivery of GTPs by subcutatneous polymeric implants decreases the effective dose dramatically while eliciting a greater biological effect as compared to the traditional oral route. Further, mechanistic studies of poly E, at submicromolar plasma levels achieved in this study, suggest that its efficacy at inhibiting BP-induced DNA damage was not a result of modulation of metabolic or DNA repair pathways but from direct scavenging of the electrophillic metabolites. Thus, subcutaneous polymeric implants may provide a viable sustained release system for chemotherapeutic agents with poor oral bioavailability such as GTPs.

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## Abbreviations

| | |
|---|---|
| **BP** | Benzo[*a*]pyrene |
| **anti-BPDE** | anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide |
| **9-OH-BPE** | 9-OH-benzo[*a*]pyrene-4,5-epoxide |
| **GTPs** | green tea polyphenols |
| **poly E** | polyphenon E |
| **EC** | (−)-epicatechin |
| **EGC** | (−)-epigallocatechin |
| **ECG** | (−)-epicatechin gallate |
| **EGCG** | (−)-epigallocatechin gallate |
| **qPCR** | real-time quantitative polymerase chain reaction |
| **LC/MS** | liquid chromatography-mass spectrometry |

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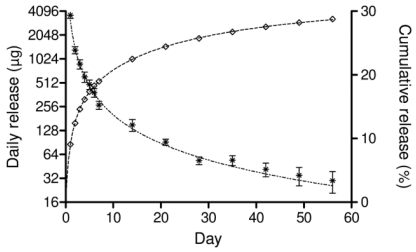


**Figure 1.**

Release of polyphenon E from polymeric implants *in vitro*. Implants (2 cm, 200 mg containing 40 mg polyphenon E) were suspended in 5 ml phosphate-buffered saline containing 10% bovine serum in a shaking water bath at 37°C. The release medium was changed daily and the amount of polyphenon E catechins released was measured as described in text.

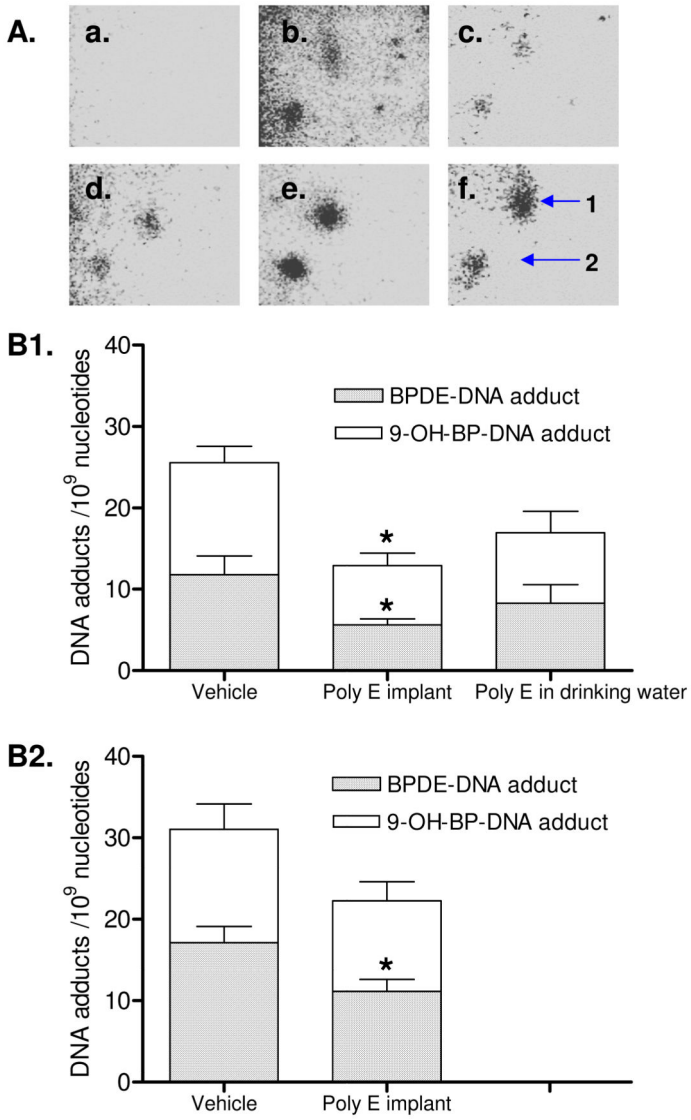


**Figure 2.**

DNA adducts in lung tissue. A, Representative autoradiographs of $^{32}$P-postlabeling analysis of benzo[*a*]pyrene (BP)-induced DNA adducts: a, one-week sham implant; b, one-week BP implant; c, one-week BP implant + polyphenon E implants; d, one-week BP implant + polyphenon E in drinking water; e, four-week BP implant; f, four-week BP implant + polyphenon E implants. 1, *anti*-benzo[*a*]pyrene-7,8-diol-9,10-epoxide-dG; and 2, 9-OH-benzo[*a*]pyrene-4,5-epoxide-dG. B1: DNA adduct levels after 1 week; and B2, DNA adduct levels after 4 weeks. *p<0.05.

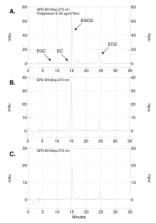

**Figure 3.**
HPLC profile of polyphenon E catechins. A, reference polyphenon E; B, extract from freshly prepared polyphenon E implants; and C, extract from polyphenon E implants recovered from the animals after 4 weeks of treatment.

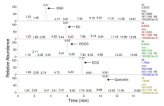

**Figure 4.**
Representative LC/MS spectrum of plasma of S/D rat treated with benzo[*a*]pyrene implant and polyphenon E implants for one week.

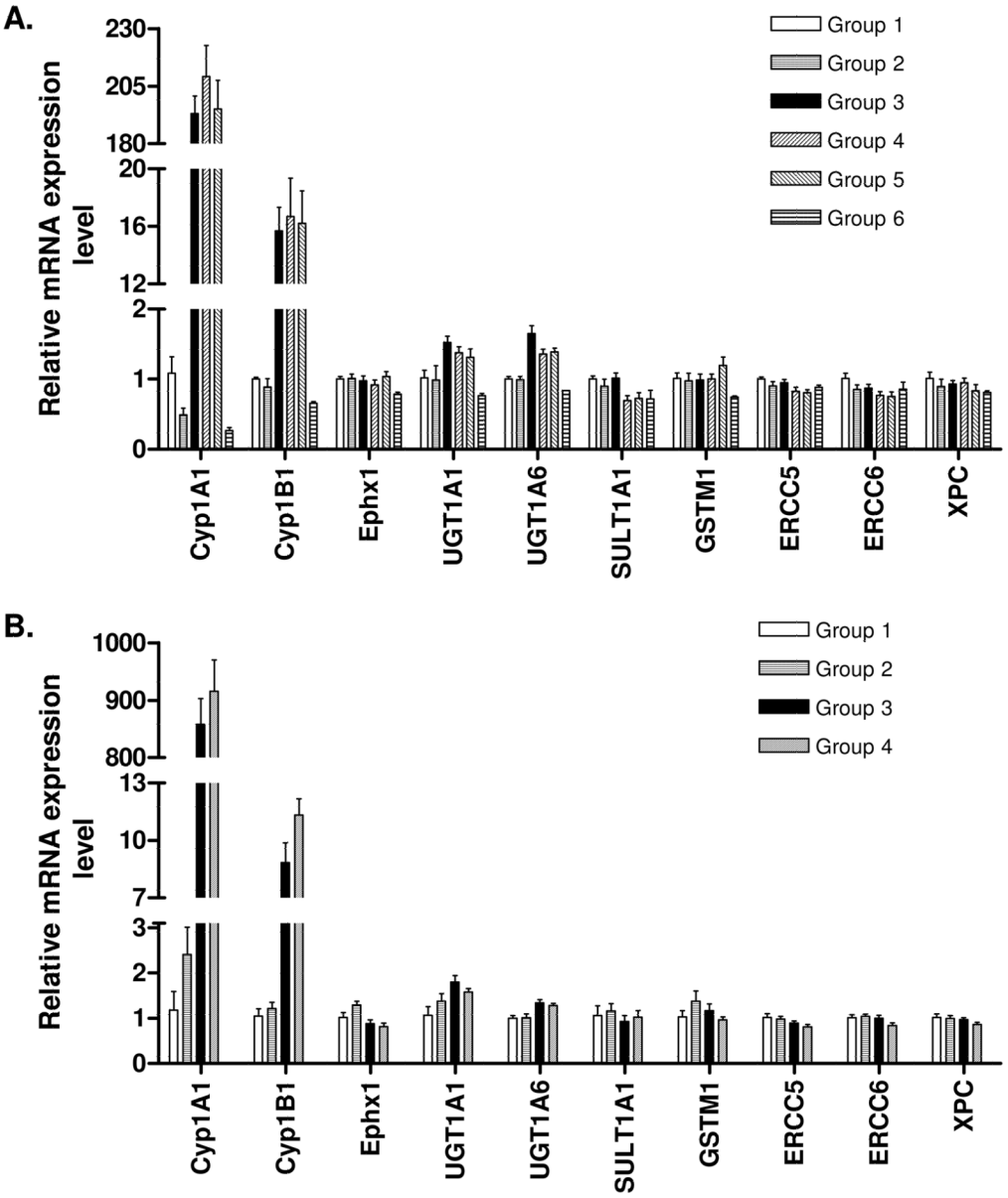


**Figure 5.**

mRNA expression of CYP1A1, 1B1, Ephx1, UGT1A1, UGT1A6, SULT1A1,GSTM1, ERCC5, ERCC6 and XPC in lung tissue of S/D rats treated with benzo[*a*]pyrene or polyphenon E or combination and indicated controls. Relative expression after 1 (A) or 4 (B) weeks of benzo[*a*]pyrene treatment. Group 1, No treatment; Group 2, Sham implants; Group 3, BP implant; Group 4, BP implant + poly E implants; Group 5, BP implant + poly E in drinking water; and Group 6, Poly E implants.

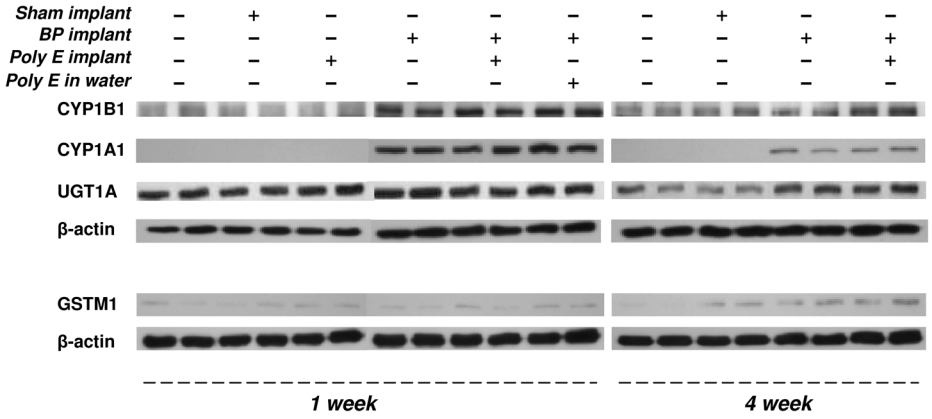


**Figure 6.**
Protein expression of CYP1A1, CYP1B1, UGT1A and GSTM1 in the lung of S/D rat treated with benzo[a]pyrene and polyphenon E as analyzed by western blotting.

## Table 1

Plasma concentrations of individuals GTPs in S/D rats treated with polyphenon E (poly E) via polymeric implants or the drinking water together with benzo[a]pyrene implant for indicated periods. Poly E catechin levels were measured by LC-MS. Data presented represent mean from five animals ± SD.

| Poly E catechins | Plasma poly E catechin levels (ng/ml) | | |
|---|---|---|---|
| | BP implant + poly E implants (1 wk) | BP implant + poly E in drinking water (1 wk) | BP implant + poly E implants (4 wk) |
| EGC | 13.3 ± 3.8 | 277.8 ± 225.3* | 7.2 ± 1.5 |
| EC | 8.8 ± 2.6 | 336.5 ± 300.9* | 3.4 ± 0.3 |
| EGCG | 60.6 ± 25.4 | 96.9 ± 43.9 | 15.2 ± 6.3 |
| ECG | 8.2 ± 4.3 | 15.8 ± 8.3 | 0.9 ± 0.9 |

* $p<0.05$

## Table 2

Enzymatic activity of CYP1 and GST following treatment of S/D rats with benzo[*a*]pyrene (BP) implant or polyphenon E (poly E) implants or combination.

| | Treatment | CYP1 activity(fold change) | GST activity(fold change) |
|---|---|---|---|
| 1 week | BP implant | 38.74 ± 7.69 | 1.17 ± 0.14 |
| | BP implant + Poly E implant | 41.21 ± 6.37 | 1.18 ± 0.17 |
| | BP implant + Poly E in drinking water | 51.15 ± 8.89 [a] | 1.21 ± 0.11 |
| | Poly E implant | 0.61 ± 0.11 | 1.23 ± 0.03 |
| 4 week | BP implant | 33.72 ± 12.39 | 1.34 ± 0.20 |
| | BP implant + Poly E implants | 23.28 ± 7.88[b] | 1.23 ± 0.22 |

Enzymatic activity was expressed as fold change of sham treatment.

[a] $p<0.05$ (BP implant + Poly E in drinking water group versus BP implant group at 1 week)

[b] $p<0.05$ (BP implant + Poly E implant 1week versus 4 week)