RESEARCH COMMUNICATION

Elimination of Deleterious Effects of Free Radicals in Murine Skin Carcinogenesis by Black Tea Infusion, Theaflavins & Epigallocatechin Gallate

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Abstract

In recent years, numerous reports have been published on the identification of novel, naturally occurring antioxidants from plants, animals, microbial sources and processed food products. Most natural antioxidants are phenolic compounds, which have a modulatory role on physiological functions and biotransformation reactions involved in the detoxification process, thereby affording protection from cytotoxic, genotoxic and metabolic actions of environmental toxicants. As part of our program on evaluation of food, beverage and traditional medicinal plants for their anticarcinogenic activity, the present report deals with the evaluation of aqueous infusion of Black tea (Camellia sinensis), Black tea extract (80% Theaflavins) & EGCG on mice exposed to the chemical carcinogen DMBA. All the four detoxification enzymes studied viz, GST, GPx, SOD and CAT were found to be activated to different degrees following treatment with black tea and two of its active compounds. The activation of the enzymes was accompanied by significant reduction in lipid peroxidation. The effect on apoptosis and cell proliferation was also studied in mice skin following administration of DMBA. Theaflavins, and EGCG significantly inhibited cell proliferation and induced apoptosis. The observation suggests chemopreventive potential of black tea infusion, black tea extract Theaflavins and the compound EGCG.

Key words: Theaflavin - Epigallocatechin gallate - Glutathione-S-transferase - Glutathione peroxidase - Superoxide dismutase - Catalase - apoptosis - cell proliferation - Dimethyl benz(a)anthracene - Chemoprevention

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Introduction

Free radicals are " any species capable of independent existence that contain one or more unpaired electrons" (Halliwell and Gutteridge 1989). In biological systems, free radicals are generated through the xenobiotic metabolism, UV and ionizing radiation, and secretion of oxidants from inflammatory leukocytes (Bland, 1986, Pryor, 1984). Because of their very high chemical reactivity, free radicals are able to induce cellular damage in a variety of ways. (Bendich, 1990) The most deleterious effects of free radicals are damage to DNA (Richter, 1988), which is associated with the process of carcinogenesis.

The process of carcinogenesis passes through multiple stages of biochemical and molecular alterations in target cells. Skin carcinogenesis involves the stepwise accumulation of genetic changes, ultimately leading to malignancy (DiGiovanni, 1992; Slaga et al., 1995; 1996). The three main steps in multistage skin carcinogenesis are (1) initiation, which involves free radicals induced genetic changes. (Garner, 1998). (2) a much slower stage of carcinogenesis is promotion, which is believed to involve selective and sustained hyperplasia, leading to the specific expansion of initiated cells into papillomas (Slaga et al., 1996) and (3) tumor progression , characterized by a high level of genetic instability that lead to chromosomal alterations (Warren et al., 1993).

To avoid such deleterious effects, the biological system have well developed efficient and widely distributed defensive enzyme system, which include glutathione S transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), which can eliminate

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and/or detoxify the free radicals. The damaging effect of free radicals can also be reduced by the natural or synthetic antioxidants.

Antioxidants can terminate the free radicals chain reaction by donating Hydrogen or electrons to free radicals and converting them to more stable products. Thus antioxidants may either delay or inhibit the initiation step of carcinogenesis. In the promotional stage of skin carcinogenesis, stem cells in the basal layer of epidermis divide more frequently, leading to formation of pre malignant lesion. This step can be reversed by the anti promoter or growth inhibitors or by the induction of apoptosis.

Tea (*Camellia sinensis*) is one of the most widely consumed beverages. Tea polyphenols have superior antioxidant effects compared to any other antioxidant present in fruits and vegetables, known to mankind (Jhawar 2000). Tea beverages are primarily manufactured as Green, Black or Oolong tea according to the degree of fermentation involved. Non-fermented green tea polyphenols have been suggested to be novel chemopreventive compounds, which can reduce UV, induced skin cancer risk in human population (Katiyar etal 2000). Flavanols are the major polyphenolic fraction in tea. Tea flavanols are commonly known as tea catechins. Some major tea catechins are (-)-epi-gallocatechin-3-gallate (EGCG), (-)-epigallocatechin(EGC), (-)-epicatechin-3-gallate (ECG), (-)-epicatechin(EC), (+)-gallocatechin (GC) and catechin (C) (Graham, 1992). In the manufacture of black tea, the "fermentation" process causes green tea catechins to oxidize and form oligomeric flavanols, including theaflavins, thearubigin and other oligomers. Theaflavins are a mixture of theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3'-gallate (TF-2b) andtheaflavin-3, 3'-digallate (TF-3). In 1983, Conney et al. were the first to demonstrate that hydroxylated flavonoids in tea had a potent inhibitory effect on mutagenic activity (Huang et al., 1983). Sugimura and his colleagues were first to use a two-stage skin carcinogenesis mouse model to demonstrate that topical application of EGCG inhibited tumor promotion induced by teleocidin in 7,12-dimethyl-benz(a)anthracene (DMBA)-initiated mouse skin (Yoshizawa et al., 1987). Khan et al., (1988) further showed that green tea polyphenols had a potent inhibitory effect on skin tumorigenicity in Sencar mice. In recent years, many studies demonstrated that topical application or oral feeding of a polyphenolic fraction from tea extract, and individual catechin derivatives, had anticarcinogenic effects in animal skin experiments (reviewed by Yang and Wang, 1993 and Katiyar and Mukhtar 1997b). Topical application or oral feeding of a green tea polyphenol fraction or its major component, EGCG, inhibited tumor initiation induced by DMBA and benzo(a)pyrene. While most of the earlier studies involved green tea polyphenols in recent years interest has shifted to black tea also. Lu et al. (1997) reported that oral administration of black tea in tumor-bearing mice inhibited proliferation and enhanced apoptosis in nonmalignant and malignant skin tumors. Chen and Ho (1994) extensively investigated the antioxidative properties of various tea polyphenols. Their study showed that the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging ability of various tea polyphenols was EGCG > ECG > EGC > EC = TF-2 > TF-1 > TF. The DPPH radical-scavenging activity was proportional to the number of -OH groups in the catechins or theaflavins. The superoxide-scavenging activity of the catechins was EGCG > ECG > EGC > EC. All the theaflavins exhibited the same ability to inhibit the\ production of superoxide. The lipid oxidation-inhibition activity of the catechins was also EGCG > ECG >EGC > EC

All these finding suggests a mechanistic link between tea components and the possible prevention of skin carcinogenesis. In this study we investigated the effect of black tea infusion, black tea extract (80% theaflavins) and EGCG on DMBA induced murine skin carcinogenesis model.

Materials and Methods

1. Experimental animals

Adult (5-6 weeks) Swiss albino male mice (23±2 gm), bred in the animal colony of Chittaranjan National Cancer Institute, Kolkata, used for the study, were maintained at controlled temperature under alternating light and dark conditions. Standard food pellets (Lipton India Ltd.) and drinking water were provided ad libitum.

2. Test materials

25 commercial brand of Black tea, available in Indian market were analyzed for the quantitative measurement of theaflavins and catechins by HPLC method. Considering the results obtained, "Chuapara" brand containing maximum amount of antioxidants, was selected for evaluation of the effect of infusion. Black tea samples were prepared using an aqueous extraction procedure, which simulated actual brewing condition of a cup of tea. Black tea extract, containing 80% theaflavins and EGCG were purchased from Sigma chemicals.

3. Carcinogenesis model

9,10-dimethyl benz(a)anthracene (DMBA, Sigma Chemicals) was applied topically in a shaved portion on the dorsal surface of the mice, at a dose of 1mg / 100 µl acetone / mouse twice at an interval of three days. This was followed by application of 1% croton oil on the same place twice weekly for 8 weeks.

4. Experimental groups

The Carcinogen control group received DMBA and croton oil and only distilled water ip in place of treatment. In the treatment groups, the animals received carcinogen as in first group but were treated with the selected doses of EGCG and theaflavins administered ip or received black tea infusion orally. Number of animals in each group was 40 of which 30 were sacrificed for histological and biochemical studies and the remaining 10 were observed

for development of skin papillomas.

5. Dose Selection

For the selection of dose, at first, the amount of EGCG and Theaflavins of very popular 25 commercial brands (data not submitted) of black tea available in Indian market were quantitatively measured. The average content of EGCG and theaflavins in 4 cups (100ml each) of 2% tea was estimated to be 27.2 & 57.28 mg. This was used as the dose selection criteria for the experiments considering the body weight of mice.

6. Detection of Papillomas

Animals in all experimental groups were continuously examined for detection of papillomas. Morphological observation was confirmed by histopathology of the growth.

7. Biochemical Estimations

Glutathione-S-transferase (GST) activity was measured in the liver cytosol after 15 days of treatment following the method of Habig et al (1974). The enzyme activity was determined from the increase in absorbance at 340 nm with 1-chloro-2-4-dinitrobenzene (CDNB) as the substrate and specific activity of the enzyme expressed as formation of 1-chloro-2-4-dinitrobenzene (CDNB) -GSH conjugate per minute per mg of protein. Glutathione peroxidase (GPx) activity was also determined after 15 days of treatment in the post mitochondrial fraction by the method of Paglia and Valentine (1967). The reaction mixture contained NADPH and glutathione reductase. The decrease in absorbance following addition of H_2O_2 was recorded at 340 nm. Enzyme activity was expressed as nmoles of NADPH utilized per minute per mg protein using molar extinction co-efficient at 340 nm as 6200 m-1cm-1. Activity of catalase (CAT) in liver was estimated by the method of Luck (1963). The enzyme activity was determined spectrophotometrically at 250 nm and expressed as unit/mg protein where the unit is the amount of enzyme that liberates half the peroxide oxygen from H_2O_2 in 100 seconds at 25°C. Superoxide dismutase (SOD) activity was determined by quantification of pyrogallol auto oxidation inhibition by the method of Marklund and Marklund (1974) and expressed as unit/mg protein. One unit of enzyme activity is defined as the amount of enzyme necessary for inhibiting the reaction by 50%. Auto oxidation of pyrogallol in Tris-HCL buffer (50mM, pH 7.5) is measured by increase in absorbance at 420 nm. Lipid peroxidation was estimated in liver microsomal fraction by using the method of Okahawa et al (1979). The level of lipid peroxides formed was measured using thiobarbituric acid and expressed as thiobarbituric acid reactive substance (TBARS) formed per mg protein using extinction co-efficient of 1.56 x 105 M-1cm-1. Protein was estimated by the method of Lowry (1951).

The level of cell proliferation was studied in situ following incorporation, of 5-bromo-2'-deoxy-uridine (BrdU) using BrdU Labeling and Detection Kit II, AP (Roche) as per kit protocol. The tissue samples were then evaluated in phase-contrast microscope. The percentage of apoptosis was measured by the TUNEL method using in situ cell death detection kit, Fluorescein (Roche). The skin tissues were fixed with 4 % paraformaldeyde. The tissue section were permeabilized with 0.1% Triton X-100 in 0.1% Sodium citrate and labeled with TUNEL reaction mixture, and observed, using fluorescence microscopy.

Results

It can be noted from the Table-1 that maximum inhibition of incidence as well as multiplicity of papillomas was produced by treatment with theaflavins followed by EGCG and black tea infusion treatment.

GST involved in initiating detoxification, was found to be enhanced following treatment with black tea infusion, black tea extract and EGCG during the early stage (15 days) of carcinogen exposure, but not in the later stages (Fig-1). A similar trend was noted in case of GPx activity, which is involved in elimination of reactive oxygen species that produce cellular damages associated with carcinogenesis (Fig-2). SOD, involved in removing the superoxides that cause oxidative damage to cells, was also found to be enhanced following all treatment during the early stage (15 days) of carcinogen exposure, but not in the later stages (Fig-3). Catalase, another detoxification enzyme, was found to be enhanced following treatment during the later stages (12

Table 1. Effect of of Black Tea Infusion and Its Active Compounds Theaflavins and Epigallocatechin Gallate in DMBA Induced Murine Skin Carcinogenesis

Groups	No. of papilloma bearing mice	Incidence of Papilloma	No of papiloma per papilloma bearing mouse	Inhibition of multiplicity (%)
Normal	0 (out of 10)	(—)	(—)	(—)
Carcinogen Control	7 (out of 10)	70.00%	3.20	0.00
2% Tea infusion	5 (out of 10)	50.00%	1.50	66.50
EGCG treated	4 (out of 10)	40.00%	2.00	64.28
Theaflavins treated	4 (out of 10)	40.00%	1.50	73.20

Inhibition of multipliticity = $\frac{\text{(Total no. of papilloma in carcinogen control) - (Total no. of papilloma in treated) X 100}}{\text{Total no. of papilloma incarcinogen control}}$

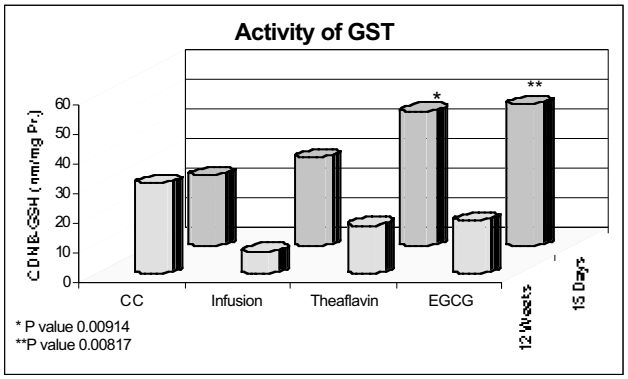

Figure 1. Glutathione-S- Transferase in Liver.

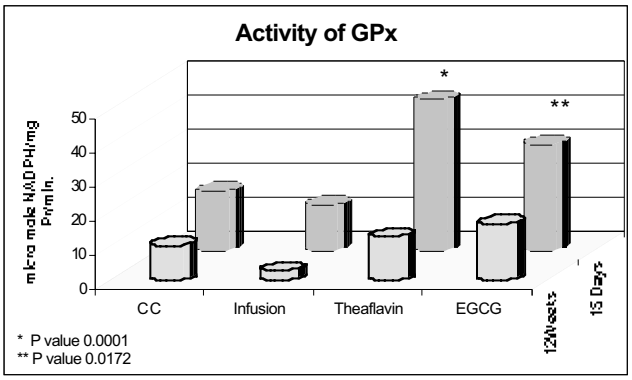

Figure 2. Activity of Hepatic Glutathione Peroxidase.

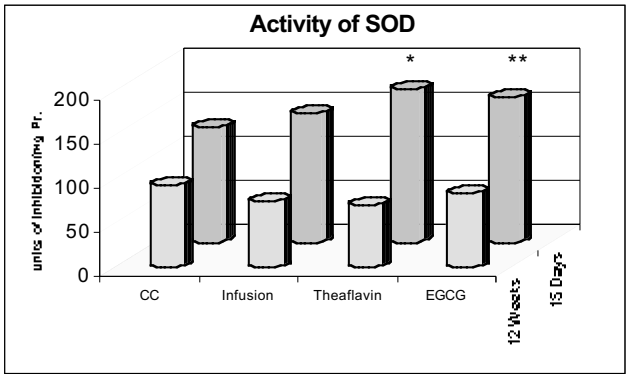

Figure 3. Superoxide Dismutase Activity in Liver.

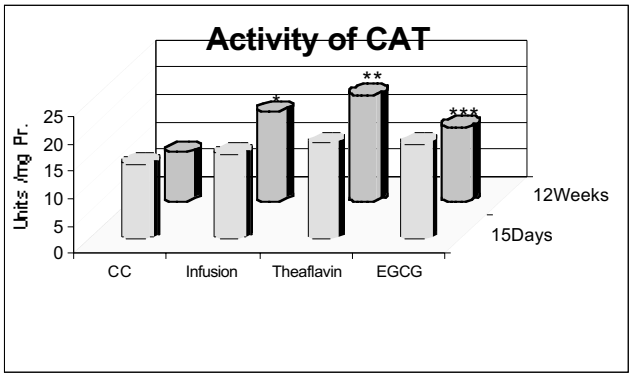

Figure 4. Activity of Catalase in Liver .

weeks) of carcinogen exposure, but not in the early stages (15 days), as noted in cases of GST, GPx and SOD (Fig-4).

Exposure to the carcinogen was found to enhance lipid peroxidation in liver. After 12 weeks of treatment with black tea, lipid peroxidation was found to be decreased significantly. The effect was most pronounced with 2% tea infusion (Fig-5).

Black tea infusion, administered orally, failed to produced

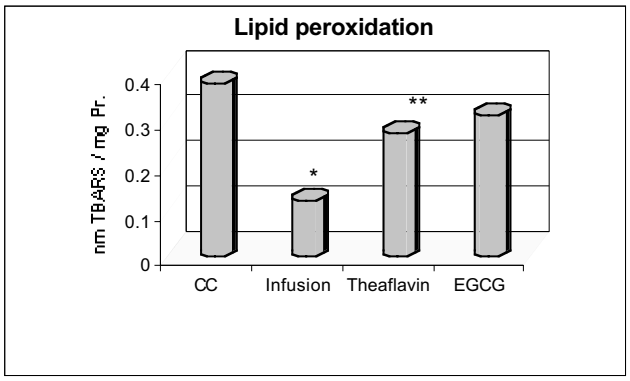

Figure 5. Effect of Black Tea Infusion and Its Active Components Theaflavins and Epigallocatechin Gallate on Lipid Peroxidation in Liver during Skin Carcinogenesis in Mice.

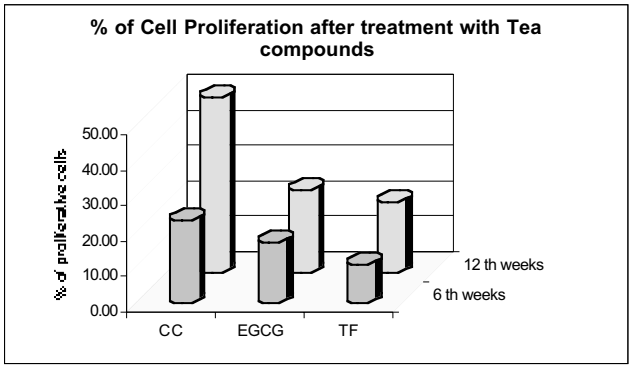

Figure 6. Effect of Black Tea Infusion, Theaflavins and Epigallocatechin Gallate on Cell Proliferation in Skin during Skin Carcinogenesis on the 6th and 12th Weeks.

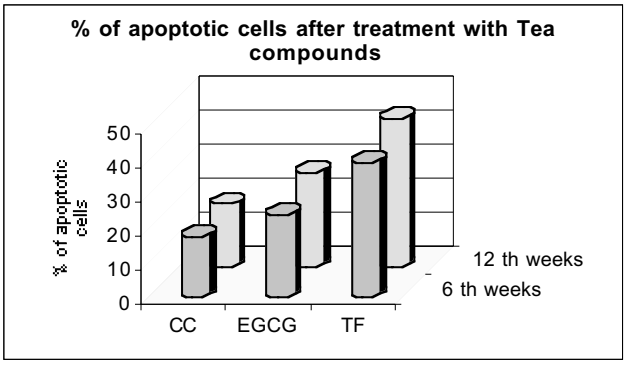

Figure 7. Effect of Black Tea Infusion, Theaflavins and Epigallocatechin Gallate on Apoptosis in Situ in Mouse Skin after 6 and 12 weeks of Carcinogenic Exposure.

any change in cell proliferation and apoptosis. But black tea extract (Theaflvins) and EGCG was found to inhibit cell proliferation (Fig-6) and induced apoptosis (Fig-7) at the target site i.e. skin epithelium.

Discussion

Current research shows tea contains some bioactive compounds including specific antioxidants and health promoting ingredients, which are implicated in lowering the risk of heart diseases, stroke and certain types of cancer. There is now a reasonable body of literature that support the concept of a chemopreventive approach for prevention of cancer by tea (Camellia sinensis).

GST plays an important role in initiating detoxification by catalyzing the conjugation of GSH to the electrophilic foreign compounds for their elimination from the system, thereby providing cellular protection against a wide variety of xenobiotics (Sedlack etal, 1968,; Szarka and Pfeiffer 1995). GPx, SOD and CAT form a part of the crucial processes involved in cellular antioxidant defense mechanism whereby peroxides and superoxides are inactivated (Vang, et al 1997). The present study demonstrates that both black tea extract and EGCG can activate these enzymes following exposure to the carcinogen DMBA that was found to result in decreased activity of these enzymes. The activation of these enzymes is also accompanied by a reduction in lipid peroxidation, a process known to generate reactive oxygen species that is associated with tissue injury and damage of cellular macromolecules (Chung etal 1996 and Wiseman etal 1996).

A single mutated cell can establish itself among a population of normal cells if the mutation select for an advantage that involve factors which modulate cell proliferation, the ability to divide faster, or ability to survive longer. In mouse epidermis 5-12 % of cells in basal layer are stem cells (Potten, 1992). The cell progeny generate a discrete column of cells from basal cells to keratinized cells, arranged in a hexagonal pattern and called an epidermal proliferative unit (Potten, 1981). Increases in epidermal proliferation by mutated stem cells, can lead to the establishment and development of a mutated clone which ultimately can result in papillomas. Cell death is a part of normal physiology for most metazoan species. During development, unwanted cells are removed through programmed cell death, making important contributions to morphogenesis, organogenesis, and other processes (Vaux and Korsmeyr, 1999). Defects in the cell death machinery, which prevents the programmed turn over of cells, can increase undesired cell accumulation, genetic instability, enhance cell longevity and permit cells to survive in a suspended state. Thus defective apoptosis may indirectly promote cancer. Treatment with both EGCG and Theaflavins resulted in inhibition of cell proliferation and induction of apoptosis at the target site initiated with DMBA.

The present study conducted in a mouse skin carcinogenesis model clearly reveal that black tea as drink as well as the major components Theaflavins and EGCG can effectively reduce the incidence and multiplicity of skin papilloma, a precancer condition which preceeds development of cacinomas. Further this study demonstrates that the functional action of black tea and its components is mediated by varied pathways, which includes activation of detoxification and prevention of cellular damage, inhibition of cell proliferation and induction of apoptosis. Similar observation was also made in our laboratory on a rat colon carcinogenesis model (Sengupta et al 2002).

In view of these observations it is suggested that although Green tea had received all the attention so far as a health promoting drink, Black tea also is a promising candidate for prevention of diseases involving oxidative damages, including cancer. Human intervention studies are therefore required to establish the cancer preventive role of this popular and widely consumed beverage.

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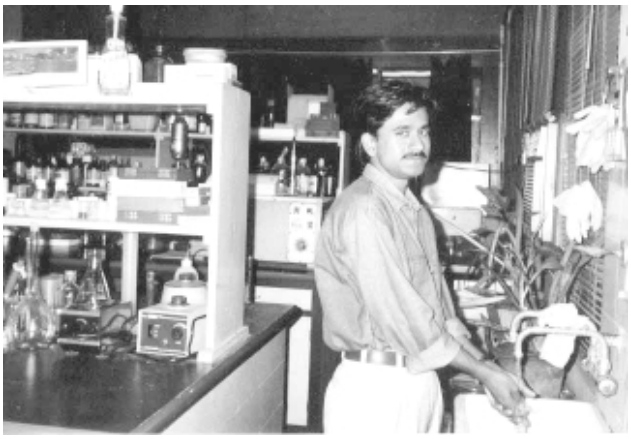

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