

## RESEARCH COMMUNICATION

# Inhibition of Cell Proliferation and Induction of Apoptosis During Azoxymethane Induced Colon Carcinogenesis by Black Tea

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### Abstract

Tea (*Camellia sinensis*) is one of the most popular beverages, consumed worldwide. The health promoting properties of tea have been attributed to its antioxidative polyphenolic constituents and their oxidative products. The aim of the present study was to evaluate the chemopreventive efficacy of a black tea infusion on azoxymethane induced colonic preneoplastic lesions, the aberrant crypt foci in Sprague-Dawley rats. Rats were injected with azoxymethane (15mg/kg.b.w.) and received oral administration of 1% and 2% (w/v) tea infusions from the 1<sup>st</sup> day of carcinogen application. The treatment was continued for 12 weeks. The colons were then assessed for aberrant crypt foci and compared with the untreated carcinogen control group. In situ cell proliferation and in situ apoptosis were also estimated using Brdu incorporation and the TUNEL method, respectively. Aberrant crypt foci were reduced significantly (by 44% in the 1% tea-treated and by about 40% in 2% tea-treated group). Significant decrease in proliferation and increase in apoptosis suggest a possible interplay between the two processes resulting in inhibition of colon carcinogenesis by black tea.

**Key Words:** ACF - azoxymethane - apoptosis - brdu - cell proliferation - TUNEL

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### Introduction

Colorectal cancers are the third most frequent cancers in the world, affecting both sexes (Kinzler, et al., 1996) and without preventive intervention, a major portion of the population is likely to develop colorectal cancer at some point during their lives.

A variety of studies have shown that colon cancers stem from nutritional traditions (Moon, et al., 1992) and many foods, beverages and their components affect the risk of developing colon cancer (Yamane et al., 1991; Wargovich et al., 1996; Steele et al., 1994; Weisberger, 1998).

Growing attention is being focussed on food components with potential cancer inhibiting effects, in the hope of identifying chemopreventive diets or dietary supplements for human use. Among potentially chemopreventive food components, polyphenolic flavonoids, characterized by hydroxylated aromatic rings that are ubiquitously present in foods of plant origin, have long been recognized to possess anticarcinogenic properties (Mutoh, 2000).

Tea, the most popular and widely consumed beverage of the world, shows anticarcinogenic action attributed to the presence of four major antioxidant flavanols: epicatechin (EC), epicatechingallate (ECG), epigallocatechin (EGC) and epigallocatechingallate (EGCG). Further, recent studies show that both green and black tea contain other flavonoids, such as, quercetin, kaempferol and myricetin which are potentially anticarcinogenic (Herlog et al., 1993; Mukhtar et al., 1988; Challa et al., 1997). Moreover, theaflavins and thearubigins, major components of black tea also possess antioxidant properties (Halder et al., 1998).

Although epidemiological studies have failed to obtain a consistent pattern regarding the association of black tea consumption and cancer risk (Blot et al., 1997), most experimental animal studies show tea has cancer preventive properties and these studies have been reviewed extensively in recent years (Kelloff et al., 1996; Dreosti et al., 1997; Katiyar et al., 1997; Conney et al., 1997; Yang et al., 1996, 1997; Mitscher et al., 1997; Agarwal et al., 1996).

In the present study, the inhibitory effects of 1% and 2%

black tea infusions were examined, in terms of azoxymethane (AOM) induced aberrant crypt foci (ACF) formed, during colon carcinogenesis in male Sprague-Dawley rats.

As increase in cellular proliferation is observed during carcinogenesis (Yang et al., 1996; Yoshikawa et al., 1996; Riso et al., 1988), in situ cell proliferation was assessed in colon. In situ cell death (apoptosis) was also measured to demonstrate the possible interplay between cell proliferation and apoptosis during the inhibitory action of tea infusion over the period of carcinogenesis.

## Materials and Methods

### Experimental Design

Male Sprague-Dawley rats, 3-4 weeks old (obtained from the animal colony of our Institution) were maintained in plastic cages (~ 4 rats/cage) at an ambient temperature of 22-25°C on a 12 hour light/dark cycle with access to drinking water and pellet diet (Lipton India Ltd.) ad libitum. Commercial black tea was bought from local market. Distilled water was boiled and 1% (w/v) and 2% (w/v) of tea infusions were prepared in boiled water brewing the tea leaves in hot water for five minutes.

The experiment was designed in three sets according to the experimental parameters used. Each group in a set consisted of ten rats. The control and treated rats received AOM (Sigma Chemicals Co., MO, USA) by three weekly subcutaneous injection (15mg/kg. b.w.) performed between 11am – 12 noon. Two treatment groups in each set received oral administration of 1% and 2% tea infusions, 1ml/rat/day starting from the 1<sup>st</sup> day of AOM injection and the control group received the same amount of distilled water.

### Set I: Assessment of ACF

This set was divided into three groups – carcinogen control, tea 1% and tea 2%. After 12 weeks of 1<sup>st</sup> AOM injection, the rats were sacrificed to assess the incidence of colonic ACF. The colons of the animals were placed flat between two filter papers and fixed in 10% buffered formalin for about 24 hours. This was followed by staining the colons with 2% methylene blue (Qualigens ltd.) in Ringer's solution for 3-5 min. ACF can easily be identified, after placing the colon on glass slide with mucosal side up under the light microscope at 40X magnification, by their large and elliptical luminal opening (McLellan et al., 1991). Total number of ACF and the number of ACF with 4 or more aberrant crypts (AC) were counted for each rat.

### Set II: In situ cell proliferation

Cell proliferation in colon was measured using 5-bromo-2'-deoxyuridine labelling (Davidson et al., 2000) with Brdu labelling and Detection Kit II, procured from Roche Molecular Biochemicals. For this purpose, rats were sacrificed and the colons were sliced into several parts and placed into Brdu labelling medium when the DNA of proliferated S-phase cells get labelled with Brdu. The tissue slices were fixed and processed by normal histological

procedures and the sections were subsequently incubated with an Anti-Brdu Monoclonal antibody (at 37 °C for 30 min.) which was detected by an Alkaline Phosphatase-conjugated-antimouse-immunoglobulin antibody (anti-mouse-Ig-AP). The bound anti-mouse-Ig-AP was visualized using Nitroblue Tetrazolium (NBT), an AP-substrate solution.

The Brdu Labelling Index (Brdu LI) was determined by dividing the number of labelled cells by the total cells counted and multiplying by 100.

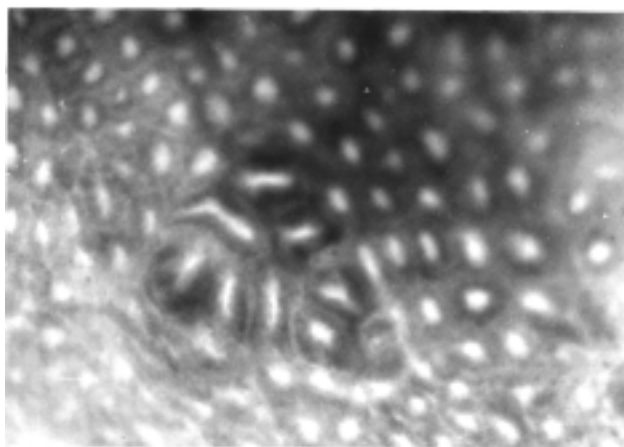
### Set III: In situ cell death detection

Apoptotic cells in colon were visualized using the terminal deoxynucleotidyl transferase (TdT)- mediated dUTP- biotin nick end labelling (TUNEL) method with the help of In situ cell detection kit, AP (Roche Molecular Biochemicals). The deparaffinized tissue sections were permeabilized using Triton-X-100 (Sigma). The sections were then incubated with TUNEL reaction mixture containing the TdT and fluorescein-dUTP, at 37 °C for 60 min. Slides were rinsed twice in PBS for 10 min. and dried around the sample; a converter AP was added and the sections were covered by a coverslip and incubated at 37 °C for 30 min. Slides were again rinsed in PBS for 10 min. and NBT was added and kept for 10-15 min. Finally, the slides were washed and analyzed under light microscope.

Apoptotic Index (AI) was determined as the percentage of the labelled nuclei with respect to the total number of nuclei counted (Caderni et al., 2000)

### Statistical Analysis

The differences in mean values among different groups were tested and the values are expressed as mean ± S.D. The data from incidences of ACF were analyzed using Students-t-test and those obtained from proliferation and



**Figure 1. Topographic View of Unsectioned Methylene Blue-Stained Mucosa of Colon from Sprague-Dawley Rat Treated with 3 Weekly s.c. Injections of AOM, Showing a Focus of 8 AC Surrounded by Normal Crypts, X 40.**

apoptotic assays were tested by analysis of variance (ANOVA). All the calculations were using MS –Excel and a p-value of <0.05 was considered significant.

**Results**

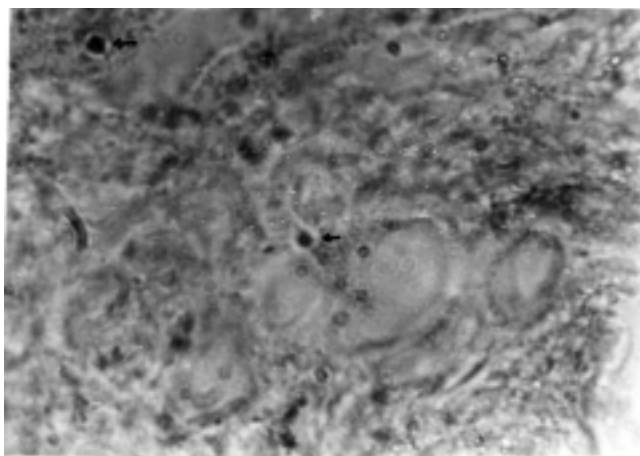
*Data for ACF (Fig. 1)*

At the end of 12 weeks treatment after 1<sup>st</sup> AOM injection, the total number of ACF and the number of large ACF with four or more aberrant crypts (AC) were found to be reduced in each treated group with respect to the corresponding carcinogen control value (Table I). Total number of ACF were significantly reduced by 44% (p= 0.0016) in 1% and

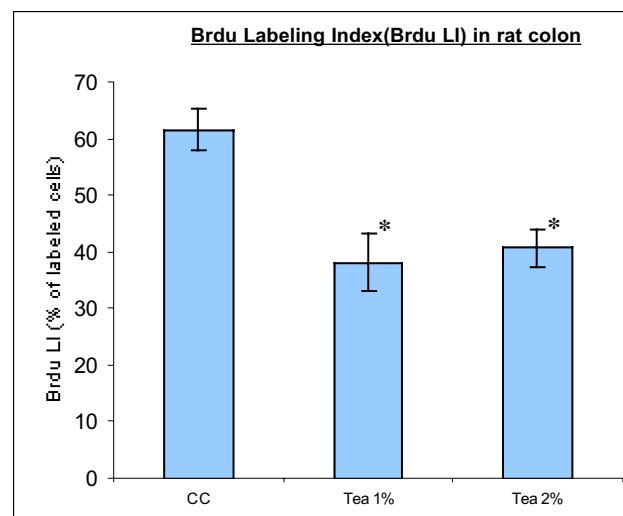
by 39.44% (p=0.0030) in 2% tea treated group. Numbers of ACF with four or more AC was reduced by 21% and 25.53% in 1% and 2% tea infusion groups respectively.

*Cell proliferation and Apoptosis (Fig. 2 and Fig. 3)*

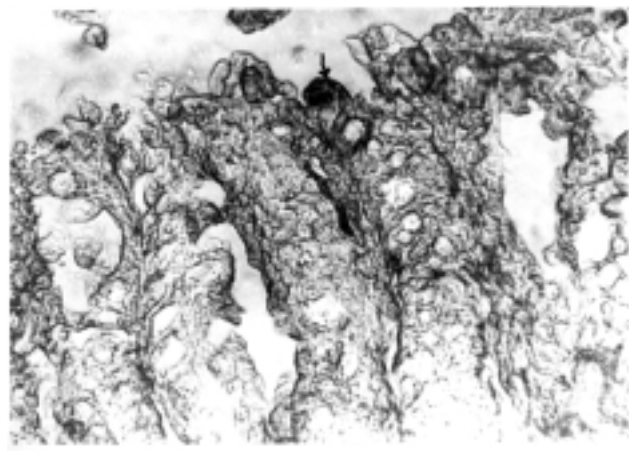
Significant reduction in Brdu LI and significant increase in AI were visualized in all the treated groups. Sections from distal 5 cm of colons were chosen and the cells were counted. The Brdu LI (Fig. 4) was found to be reduced by 37.87%



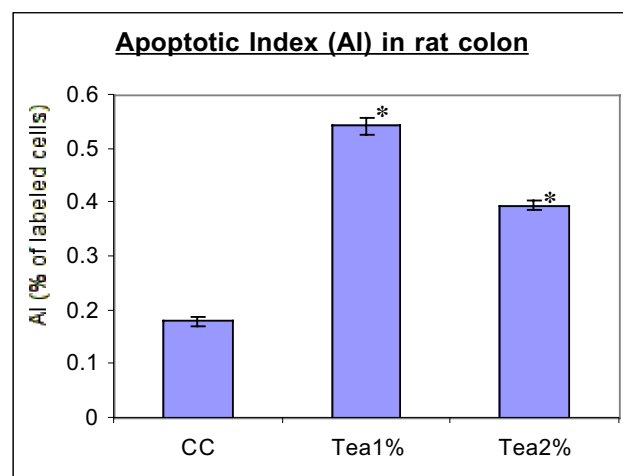
**Figure 2. Immunohistochemical Detection of Cell Proliferation using Brdu-incorporation Method. Proliferated (S-phase) Labeled Nuclei are Dark Stained**



**Figure 4. Cell Proliferation is Expressed as Brdu LI which is Calculated as Percentages of Brdu Labeled Cells with Respect to the Total no. of Cells Counted Throughout the Distal 5 cm of Colons from each Group. \*Found Statistically Significant w.r.t. the Control Value.**



**Figure 3. Immunohistochemical Detection of Apoptotic Cells using TUNEL Method. The Apoptotic Cell found within the Field has been Indicated by an Arrow, X 40.**



**Figure 5. Apoptosis is Expressed as AI, Calculated as the Percentage of TdT-labeled Cells w.r.t the Total Number of Cells Counted Throughout the Distal 5 cm of Colons from each Group. \*Found Statistically Significant w.r.t. the Control Value.**

**Table 1. Analysis of ACF in Rat Colon after 12 weeks of 1<sup>st</sup> AOM Injection.**

Groups	Total ACF Mean ± SD	% inhibition after 12 weeks	ACF(4/moreAC) Mean ± SD	% inhibition after 12 weeks
Carcinogen Control	27.3 ± 3.1	-	11.8 ± 4.2	-
Tea 1%	15.3±1.5*	44.0	9.3±1.0	21.0
Tea 2%	16.5±1.3*	39.5	8.8±1.0	25.5

Total number of ACF (mean ± SD) and the number of ACF with 4 or more AC (mean ± SD); Percentage of inhibition has been calculated w.r.t. the carcinogen control value.

\* Significantly different (statistically) from carcinogen control value.

( $p < 0.001$ ) in 1% tea treated and by 33.85% ( $p < 0.001$ ) in 2% tea treated group. AI (Fig. 5), which indicates the level of programmed cell death was found to be increased significantly in all treated groups in comparison to the carcinogen control value ( $p < 0.001$  in 1% tea and  $p < 0.01$  in 2% tea treated group).

## Discussion

Tea and its constituents have wide range of biological activities that may contribute to chemoprevention of cancer by acting on one or more sites during the process of carcinogenesis. ACF have been identified as the earliest recognizable histopathological change seen in developing neoplasia in the carcinogen-induced rodent models (Bird, 1993) and was also identified in human colonic neoplasia (Pretlow et al., 1991).

As indicated by the results obtained, it may be noted that black tea infusions were able to reduce ACF formation in AOM induced male Sprague-Dawley rats without causing any adverse effects. Further, the number of ACF containing four or more aberrant crypts was also reduced considerably showing protective effect of tea on the process of colon carcinogenesis.

Increased proliferation plays a central role during carcinogenesis and it has been suggested that a shift of proliferation towards the luminal surface of the epithelium occurs in adenomas as well as in ACF (Riso et al., 1988; Yang et al., 1996). Hence decreased number of ACF and reduction in Brdu LI seems to be correlated. In the current study, tea drinking decreased the Brdu LI in colon. Further, AI was also found to be increased in the black tea treated groups.

In the colon, enhancement of cell proliferation, expansion of cell proliferation zone and inhibition of apoptosis are considered as risk factors for tumour development as well as for multistage carcinogenesis. It has been suggested that if mutated colonic epithelial cells do not undergo apoptosis, then mutations that lead to colon cancer may be acquired and fixed through further proliferation (Jenab et al., 2000). It has been shown that apoptosis is key for the timely death of cells with damaged DNA. Suppression of apoptosis may lead to an increase in life span or to an accumulation of DNA damage (Williams

et al., 1993; Kerr et al., 1994), while promotion of apoptosis would result in the removal of damaged cells. Since it has been suggested that disruption of apoptosis may be an early event in carcinogenesis (Hoffman et al., 1994), the observation that tea infusion increase the rate of apoptosis suggests that the protective effect may be through the modulation of apoptotic pathway.

Increased proliferation and reduced apoptosis, i.e., the balance between the two processes is important in the genesis of multistage carcinogenesis (Bedi et al., 1995). Reduction in Brdu LI and increase in AI noted in our study may be responsible for inhibition of ACF formation.

During AOM induced colon carcinogenesis, AOM is ultimately metabolized into an electrophilic methyl diazonium ion which can methylate cellular nucleophiles, including DNA (Sohn et al., 1991; Talalay, 1992; Fiala et al., 1987; Tanaka et al., 1997). Inhibition of colon carcinogenesis processes by black tea infusions may be attributed to trapping of activated electrophilic forms of carcinogen by flavanol-carcinogen adduct formation in treated rats. The component from tea may modulate the activity of some enzymes, viz., Protein kinase C, DNA polymerase and cyclooxygenase (Ho et al., 1994) which are closely related to cellular proliferation. At the same time, EGCG and ECG found in tea have been shown to induce apoptosis (Okabe et al., 1997).

Both 1% and 2% tea infusions show protective effect on colon carcinogenesis during this short term study and little differences in effects between two percentages of tea leaves were observed. This may be due to almost no difference in the amount of polyphenols that come out in the infusion during its preparation. Long term bioassay is necessary to study the end point (adenocarcinoma) and also to explore the chemopreventive actions of the active components of black tea.

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