

# Chemoprevention of tea on colorectal cancer induced by dimethylhydrazine in Wistar rats

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**Subject headings** colorectal neoplasms; dimethylhydrazine; tea; apoptosis; aberrant crypt foci; rats; proliferating cell nuclear antigen; chemoprevention

Jia XD, Han C. Chemoprevention of tea on colorectal cancer induced by dimethylhydrazine in Wistar rats. *World J Gastroentero*, 2000;6(5): 699-703

## Abstract

**AIM** To investigate the chemopreventive effects of green tea and tea pigment on 1,2-dimethylhydrazine (DMH)-induced rat colorectal carcinogenesis.

**METHODS** Male weaning Wistar rats were randomly allocated into four groups. Rats in the positive control group were given s.c. injection of DMH, once a week for ten weeks; rats in tea-treated groups, with the same DMH treatment as in the positive group, received 2% green tea and 0.1% tea pigments; rats in the negative control group were given s.c. injection of the same volume of saline as well as DMH in the positive group. Animals were sacrificed and necropsied at the end of week 16 and week 32.

**RESULTS** Aberrant cryptic foci (ACF) were formed in animals in DMH-treated groups at the end of week 16. Compared to the DMH group, green tea and tea pigments groups had less ACF (148.25 and 204.25, respectively,  $P < 0.01$ ). At the end of week 32, all rats in DMH group developed large intestinal tumors. The results also showed that DMH increased labeling index (LI) of proliferating cell nuclear antigen (PCNA) of intestinal mucosa and the expression of ras-p21. However, in the tea-treated groups, PCNA-LI was significantly reduced as compared with the positive control group (36.63 and 40.36 in the green tea group and tea pigment group, respectively, at the end of the experiment,  $P < 0.01$ ). ras-p21 expression was also significantly reduced (2.07 and 2.36 in the colon tumors of rats in the green tea group and tea pigments group, respectively at the end of the experiment,  $P < 0.01$ ). Furthermore, green tea

and tea pigment inhibited the expression of Bcl-2 protein (2, 5, 1, 0 and 2, 4, 1, 0, respectively, at the end of the experiment  $P < 0.01$ ), and induced expression of Bax protein (0, 1, 3, 4 and 0, 1, 4, 3, respectively,  $P < 0.01$ ).

**CONCLUSION** Chinese green tea drinking inhibited ACF and colonic tumors formation in rats, which showed that tea had a significant chemopreventive effect on DMH-induced colorectal carcinogenesis. Such effects may be due to suppression of cell proliferation and induction of apoptosis in the intestinal crypts.

## INTRODUCTION

Colorectal cancer is the third most common malignant neoplasm worldwide<sup>[1]</sup>. In China, the incidence and mortality rates of colorectal cancer are increasing in recent decade, being the fifth leading cause of cancer deaths<sup>[2]</sup>.

Aberrant crypt foci (ACF) in the colonic mucosa have been hypothesized to represent precursor lesions of chemically induced colon cancer. Aberrant crypts can be identified by their increased size, thicker epithelial lining, and increased pericryptal zone<sup>[3]</sup>. Various studies have supported the concept that ACF are precancerous lesions that can be used as biologic end points in the study of modulators of colon carcinogenesis<sup>[4-9]</sup>.

Tea is one of the most popular beverages consumed worldwide. Tea polyphenols is the major constituent of green tea, whereas tea pigments, the major constituent of black tea, is a compound which is mainly composed of teaflavin and tearubigin. Many studies on the possible modifying effect of green tea and tea polyphenols on experimentally induced colorectal cancer have been carried out during the last decades<sup>[10]</sup>, but there are few reports about the effect of black tea and tea pigments.

Carcinogen-induced ACF formation in rodent colon has been used as a short-term bioassay to evaluate the role of nutritional elements and to screen potentially new chemopreventive agents<sup>[11]</sup>, however, few reports have been found regarding the early detection of ACF corresponding to the later development of tumors<sup>[12]</sup>. The purpose of our present study is to find out the effects of green tea and tea pigments on ACF formation and colorectal cancer induced by DMH in Wistar rats, and to appraise their possible mechanisms.

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Received 2000-05-25 Accepted 2000-06-23

## MATERIALS AND METHODS

### Materials

Green tea and tea pigments were provided by the Institute of Tea Science and Research, Chinese Academy of Agricultural Sciences. The water extract of green tea was prepared freshly everyday as follows: 2g of green tea leaves (Long Jing brand) was dissolved in 100mL boiling water, allowed to stand at room temperature for 30 minutes, and then filtered. Tea pigment solution was prepared freshly daily.

### Animals and treatment

168 weaning male Wistar rats were purchased from the Animals Breeding Center of Chinese Academy of Medical Sciences, Beijing, and were randomly allocated into four groups, 42 rats in each. The animals were maintained in a controlled environment at  $24^{\circ}\text{C}\pm 1^{\circ}\text{C}$  and  $50\%\pm 10\%$  relative humidity with an altering 12:12-hour light-dark cycle. Rats in group 1 (positive control) were given s.c. injection of DMH-2HCl (Sigma Chemical) once weekly for 10 weeks at a dose of 20mg/kg body weight. Animals in groups 2 and 3, in addition to the same carcinogen treatment as in group 1, received 2% green tea and 0.1% tea pigments, respectively, as the sole source of drinking fluid. Animals in group 4 (negative control group) were injected s.c. with equal volumes of physiological saline. Rats in group 1 and group 4 were given tap water as drinking fluid. Body weight of all animals was recorded weekly until the last DMH injection, and then every 4 weeks until the end of the study. The daily consumption of tea and water were also recorded. Animals were killed at weeks 16 and 32. The colons were rapidly removed and opened longitudinally, cleaned with cold saline, fixed in 10% formalin, dehydrated and embedded in paraffin. Five- $\mu\text{m}$ -thick sections were processed for histopathological examination for proliferating cell nuclear antigen (PCNA) analysis, and bcl-2 and bax analysis. For ras-p21 analysis, colonic mucosa were scraped with a microscope slide and stored at  $-80^{\circ}\text{C}$  until used. The number of tumors were counted and the length (L), width (W) and height (H) of each tumor were measured to calculate the tumor volume ( $V=L\times W\times H\times\pi/6$ ).

### Quantification of ACF

ACF were quantified following the protocol established by McLellan and colleagues<sup>[13]</sup>. Fixed colon specimens were stained in 0.02% methylene blue, and the number and growth of ACF were assessed under the light microscope. Criteria used to identify the ACF included: ① increased size, ② thicker epithelial cell layer, and ③ increased pericryptic zone. To determine crypt multiplicity, ACF were further categorized as small (1-3 crypts/focus), medium (4-6 crypts/focus), and large ( $\geq 7$  crypts/focus).

### Immunohistochemical analysis of PCNA

Two deparaffinized sections were used for PCNA staining by using a Straptavidin/ Peroxidase (SP) kit (ZYMED). Following the instructions, the anti-PCNA monoclonal antibody (mouse anti rat, ZYMED) was diluted with antibody-diluting buffer (1:100) and used for tissue sections.

The PCNA labeling index (LI) was determined by identifying 10 well-oriented crypts in which the base, lumen, and the apex of crypts displayed a U-shaped configuration. The PCNA-LI was calculated as the number of positive cells per crypt divided by the total number of cells per crypt multiplied by 100.

### Measurement of ras-p21 expression

The expression of ras-p21 was detected by Western blot<sup>[14]</sup>. Briefly, colonic mucosa and tumor specimens were washed in ice-cold phosphate-buffered saline (PBS) and suspended in disruption buffer. The specimens were homogenized and left on ice for 30min. The extracts were purified by centrifugation at  $12\,000\times g$  for 20min at  $4^{\circ}\text{C}$ .

Clear extracts of colonic mucosa and tumors corresponding to 100 $\mu\text{g}$  total protein were subjected to SDS-PAGE according to the method of Laemmli<sup>[15]</sup>. The separated protein was then transferred to nitrocellulose membrane and detected with mouse monoclonal antibody pan-ras. Densitometric analysis of immunoblots were then performed for quantification of each band.

### Immunohistochemical analysis of Bcl-2 and Bax

Bcl-2 and Bax were stained using the same method as PCNA staining except using the rabbit polyclonal Bcl-2 antibody (ZYMED) in 1:100 dilution and rabbit polyclonal Bax antibody (ZYMED) in 1:100 dilution.

Positive cells were quantified by two independent observers, expressed as the percentage of the total number of cells, and assigned to one of 4 categories: 1, 0% - 25%; 2, 25% - 50%; 3, 50% - 75%; and 4, 75%-100%.

### Statistical analysis

Body weight, tea consumption, number of ACF, number of tumors, PCNA-LI and ras-p21 expression among the 4 groups were compared by Student's *t* test. Tumor volumes and the grading of Bcl-2 and Bax expression were compared by the Wilcoxon-rank test.

## RESULTS

### General observations

Table 1 presents the body weight of the 4 groups of animals. At the end of week 32, then compared with the negative control group, the body weights in the other three groups (DMH-treated) decreased.

However, analysis of the data revealed no significant differences between them. In addition, the quantity of tea and water consumption in 4 the groups were also not significantly different.

### ACF formation

ACF was formed in DMH-treated groups (groups 1-3), but there was no ACF formation in the negative control group (group 4). Compared with the positive control group (group 1), the total number of ACF per colon in tea-treated groups 2 and 3 decreased significantly ( $P < 0.01$ ). Small, medium and large ACF in tea-treated groups were also significantly different from group 1 (Table 2).

### Cancer formation

The results of cancer formation are listed in Table 3. At the end of the experiment, all rats in the positive group developed colonic cancer, but none was developed in the negative control group. Animals that consumed 2% green tea and 0.1% tea pigments had significantly fewer cancer than that of the positive control group, and the mean volume also dramatically lower.

### Immunohistochemical analysis of PCNA

Table 4 shows the immunohistochemical analysis of PCNA-LI. After 16 and 32 weeks, the PCNA-LI in DMH-treated groups increased significantly over the negative control group. Oral administration of 2% green tea and 0.1% tea pigments diminished the PCNA-LI ( $P < 0.01$ ).

### Western blot analysis of ras-p21 expression

Table 5 summarizes the results of Western blot analysis for ras-p21 expression in both colonic mucosa and the cancers. At the end of weeks 16 and 32, Green tea and tea pigments significantly suppressed the expression of ras-p21 in colonic mucosa and cancer as compared with the positive control group ( $P < 0.01$ ).

### Bcl-2 and Bax expression

The results of immunohistochemical analysis of Bcl-2 and Bax expression are shown in Tables 6 and 7. At the end of weeks 16 and 32, Bcl-2 expression was significantly suppressed in tea-treated groups as compared with the positive control group, while Bax expression was significantly induced in groups 2 and 3 ( $P < 0.05$  or  $P < 0.01$ ).

**Table 1 Body weights during the study ( $\bar{x} \pm s$ )**

Treatment groups	Week 0	Week 16	Week 32
DMH	70.14 $\pm$ 5.92	425.69 $\pm$ 44.06	456.25 $\pm$ 94.36
Green tea+DMH	69.73 $\pm$ 6.25	427.60 $\pm$ 44.11	459.19 $\pm$ 95.52
Tea pigments+DMH	70.23 $\pm$ 5.35	425.39 $\pm$ 46.99	463.24 $\pm$ 98.50
Negative control	69.62 $\pm$ 6.20	430.89 $\pm$ 47.36	487.31 $\pm$ 99.58

DMH: dimethylhydrazine; EDTA: ethylene diamine tetracetate acid.

**Table 2 Effect of tea on DMH-induced ACF ( $\bar{x} \pm s$ )**

Treatment groups	Total	Small	Medium	Large
DMH	249.88 $\pm$ 10.891	61.50 $\pm$ 8.07	63.75 $\pm$ 1.85	24.62 $\pm$ 2.87
Green tea+DMH	148.25 $\pm$ 14.05 <sup>a</sup>	91.75 $\pm$ 12.63 <sup>a</sup>	44.10 $\pm$ 1.80 <sup>a</sup>	12.50 $\pm$ 1.58 <sup>a</sup>
Tea pigments+DMH	204.25 $\pm$ 11.94 <sup>a</sup>	126.50 $\pm$ 10.37 <sup>a</sup>	53.00 $\pm$ 2.24 <sup>a</sup>	17.38 $\pm$ 3.04 <sup>a</sup>
Negative control	0	0	0	0

ACF: aberrant crypts foci.

<sup>a</sup> $P < 0.01$ , comparison with DMH group by Student's *t* test.

**Table 3 Effect of tea on DMH-induced colon tumors**

Treatment groups	<i>n</i> <sup>a</sup>	<i>n</i> <sup>b</sup>	Average number of tumors per rat	Mean tumor volume
DMH	20	20	2.55 $\pm$ 1.36	294.69 $\pm$ 614.43
Green tea+DMH	20	14	1.35 $\pm$ 1.27 <sup>c</sup>	66.87 $\pm$ 58.20 <sup>d</sup>
Tea pigments+DMH	20	16	1.45 $\pm$ 1.16 <sup>c</sup>	94.83 $\pm$ 120.89 <sup>d</sup>
Negative control	20	0	0	0

<sup>a</sup>No. of animals; <sup>b</sup>No. of animals with tumors;

<sup>c</sup> $P < 0.01$ , comparison with DMH group by Student's *t* test.

<sup>d</sup> $P < 0.01$ , comparison with DMH group by Wilcoxon test.

**Table 4 Effect of tea on PCNA-LI in colonic mucosa of DMH-treated rats ( $\bar{x} \pm s$ )**

Treatment groups	PCNA-LI	
	Week 16 ( <i>n</i> =8)	Week 32 ( <i>n</i> =8)
DMH	41.75 $\pm$ 6.01	52.53 $\pm$ 5.40
Green tea +DMH	22.02 $\pm$ 3.63 <sup>a</sup>	36.63 $\pm$ 5.41 <sup>a</sup>
Tea pigments +DMH	29.66 $\pm$ 2.65 <sup>a</sup>	40.36 $\pm$ 5.64 <sup>a</sup>
Negative control	14.34 $\pm$ 4.68	15.43 $\pm$ 5.08

PCNA: proliferating cell nuclear antigen.

<sup>a</sup> $P < 0.01$ , comparison with DMH group by Student's *t* test.

**Table 5 Effect of tea on expression levels of ras-p21 ( $\bar{x} \pm s$ )**

Treatment groups	ras-p21 expression		
	Week 16 ( <i>n</i> =8)	Week 32 ( <i>n</i> =8)	Week 32 ( <i>n</i> =8)
	colonic mucosa	colonic mucosa	colon tumors
DMH	2.03 $\pm$ 0.35	2.26 $\pm$ 0.28	3.16 $\pm$ 0.32
Green tea +DMH	1.36 $\pm$ 0.14 <sup>a</sup>	1.48 $\pm$ 0.12 <sup>a</sup>	2.07 $\pm$ 0.15 <sup>a</sup>
Tea pigments +DMH	1.51 $\pm$ 0.19 <sup>a</sup>	1.72 $\pm$ 0.15 <sup>a</sup>	2.36 $\pm$ 0.16 <sup>a</sup>
Negative control	1	1	1

<sup>a</sup> $P < 0.01$ , comparison with DMH group by Student's *t* test.

**Table 6 Effect of tea on Bcl-2 expression**

Treatment groups	Week 16 ( <i>n</i> =8)				Week 32 ( <i>n</i> =8)			
	I	II	III	IV <sup>a</sup>	I	II	III	IV
DMH	1	1	3	3	0	1	2	5
Green tea +DMH	2	5	1	0 <sup>b</sup>	2	5	1	0 <sup>c</sup>
Tea pigments+DMH	1	5	1	1 <sup>b</sup>	2	4	2	0 <sup>c</sup>
Negative control	6	2	0	0	5	3	0	0

<sup>a</sup>Positive cells were expressed as the percentage of the total number of cells, and classified into 4 categories: I, 0%-25%; II, 25%-50%; III, 50%-75%; and IV, 75%-100%.

<sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ , comparison with DMH group by Wilcoxon test.

**Table 7 Effect of tea on Bax expression**

Treatment groups	Week 16 ( <i>n</i> =8)				Week 32 ( <i>n</i> =8)			
	I	II	III	IV <sup>a</sup>	I	II	III	IV
DMH	2	5	1	0	3	5	0	0
Green tea +DMH	0	1	5	2 <sup>b</sup>	0	1	3	4 <sup>c</sup>
Tea pigments+DMH	0	3	4	1 <sup>b</sup>	0	1	4	3 <sup>c</sup>
Negative control	6	2	0	0	5	3	0	0

<sup>a</sup>Positive cells were expressed as the percentage of the total number of cells, and classified into 4 categories: I, 0%-25%; II, 25%-50%; III, 50%-75%; and IV, 75%-100%.

<sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ , comparison with DMH group by Wilcoxon test.

## DISCUSSION

It is recognized that colon carcinogenesis is a multistep process that includes sequential selection and propagation of preneoplastic lesions. ACF are present in carcinogen-treated rodent colons as well as in humans at high risk for colon cancer development and in patients with colon cancer<sup>[16,17]</sup>. Several studies investigating the genotypic, morphological, and growth features of ACF have supported the contention that ACF are preneoplastic lesions<sup>[4]</sup>. The ACF system is frequently used to identify and study the modulation of colon carcinogenesis. The results of the present study indicated drinking 2% green tea and 0.1% tea pigments significantly inhibited the formation of ACF, and decreased significantly the numbers and size of tumor. Some epidemiologic studies revealed an inhibitory effect of green tea on the incidence of colorectal cancer<sup>[18,19]</sup>. In addition, many experimental studies have demonstrated that green tea and tea polyphenols have significant inhibitory effects on rodent colorectal carcinogenesis<sup>[20]</sup>. Our study also demonstrated that 2% green tea significantly inhibited DMH-induced ACF formation and colorectal cancer. Black tea consists of significant amount of tea pigments including teaflavins, tearubigins, etc. Teaflavins have antioxidative and antimutagenic effects<sup>[21]</sup>. However, very few studies on animal tumorigenesis model have been reported. Morse *et al*<sup>[22]</sup> demonstrated that teaflavins in drinking water reduced esophageal tumor induced by N-nitrosomethylbenzylamine (NMBzA) in rats. We have observed that tea pigments have significant inhibitory effects on oral carcinogenesis<sup>[23]</sup>. The present study demonstrated that as little as 0.1% tea pigments could significantly inhibit colorectal tumor induced by DMH in rats. It has been suggested that tea pigments may play important roles on the protective effect of black tea in chemical carcinogenesis models.

Abnormal cellular proliferation is one of crucial mechanisms in carcinogenesis<sup>[24]</sup>. PCNA is an auxiliary protein of the DNA polymerase delta, reaching an expression peak during the S-phase of the cell cycle and playing an important role in cellular proliferation<sup>[25,26]</sup>. PCNA-LI has been used as an intermediate biomarker in chemoprevention of colorectal cancer<sup>[27]</sup>. Zheng *et al*<sup>[28]</sup> observed that vitamin A significantly decreased PCNA-LI in the AOM-induced colorectal cancer animal model. Another study gave similar result<sup>[29]</sup>. In this study, both green tea and tea pigments significantly inhibited PCNA-LI at the end of weeks 16 and 32.

Recent evidence indicates that activation of ras proto-oncogenes, coupled with the loss or inactivation of suppressor genes induces a malignant phenotype in colonic cells<sup>[30]</sup>. The *ras* proto-oncogenes (c-Ki-ras, c-Ha-ras and N-ras)

constitute a family of highly conserved genes encoding a structurally and functionally related 21 kd protein, referred to as *ras*-p21, which is anchored to the cytoplasmic face of the plasma membrane, binds to the guanine nucleotides GTP and GDP. Ras activation represents one of the earliest and most frequently occurring genetic alterations associated with human cancers, especially in cancer of colon<sup>[31-34]</sup>. Elevated levels of *ras*-p21 were correlated with increased cell proliferation, histological grading, nuclear anaplasia and degree of undifferentiation<sup>[35,36]</sup>. In experiments where mutated *ras* genes are selectively inactivated, the pre-existing tumor phenotype reverts to a more normal form, indicating activated *ras* may be necessary for the maintenance of malignant behavior<sup>[37]</sup>. Recent studies have demonstrated a strong correlation between dietary modulation of carcinogen-induced *ras* activation and consequent tumor outcome<sup>[38]</sup>. Singh *et al*<sup>[39]</sup> had suggested that determination of *ras*-p21 might be a useful marker to evaluate the effectiveness of tumor inhibitory properties in colon carcinogenesis. In this study, the DMH-induced expression of *ras*-p21 was significantly suppressed both in tumors and in uninvolved colonic mucosa by oral feeding of 2% green tea and 0.1% tea pigments.

Colorectal cancer is believed to result from a series of genetic alterations that destroys normal mechanisms controlling the cell growth<sup>[30,40]</sup>. Apoptosis or programmed cell death appears to be an important mechanism in deletion of tumor cells rather than increased cell proliferation<sup>[41-43]</sup>. The *bcl-2* proto-oncogene is a known inhibitor of apoptosis and may therefore allow an accumulation of genetic alterations that become propagated by cell division and potentially contribute to neoplastic development<sup>[44-46]</sup>. The *bcl-2* gene encodes a 25 kd protein that localizes to the mitochondrial membrane, nuclear envelope, and endoplasmic reticulum<sup>[47]</sup>. Sinicrope *et al*<sup>[48]</sup> analyzed the expression of Bcl-2 oncoprotein during colorectal tumorigenesis and concluded that abnormal activation of *bcl-2* gene appeared to be an early event on colorectal tumorigenesis that can inhibit apoptosis *in vivo* and may facilitate tumor progression. Increased *bcl-2* expression could occur in conjunction with changes in the expression of other members of the *bcl-2* family, including those counteract the antiapoptotic effects of Bcl-2. One candidate in this regard is Bax, a dominant repressor of Bcl-2 that forms heterodimers with Bcl-2 and accelerates rates of cell death<sup>[49,50]</sup>. A recent study found that the development of IQ induced colorectal tumors was accompanied by the progressive inhibition of programmed cell death which was associated with increased expression of the antiapoptosis protein Bcl-2 and decreased expression of Bax<sup>[51]</sup>. In our study, the expression

of Bcl-2 was inhibited significantly by oral 2% green tea and 0.1% tea pigments, while the expression of Bax was induced significantly. The results demonstrated that green tea and tea pigments induced apoptosis.

In conclusion, the present study indicated that green tea and tea pigments significantly inhibited ACF and colorectal cancer induced by DMH, and our study further supported the hypothesis that ACF are precancerous lesions of colorectal cancer and the ACF system can be used as a short-term bioassay to screen potentially new chemopreventive agents and to evaluate the effect of protective factors at a very early stage of the carcinogenic process. Although the mechanisms of the inhibitory effects of tea preparations on carcinogen-induced colorectal carcinogenesis have not been fully elucidated, our study showed that inhibition of proliferation and induction of apoptosis may be two important mechanisms.

**ACKNOWLEDGEMENTS** We thank Dr. Qi-Kun Chen of the Institute of Tea Science and Research, Chinese Academy of Agricultural Science for his generous offer of tea samples, and are also grateful to Dr. Chung S. Yang of Rutgers University for his kind review of our manuscript.

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Edited by Wu XN

Proofread by Zhu LH and Ma JY