Chemopreventive effect of trans-resveratrol - a phytoalexin against colonic aberrant crypt foci and cell proliferation in 1,2-dimethylhydrazine induced colon carcinogenesis

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Prevention of cancer remains a primary need and new chemopreventive agents must be developed for this purpose. Towards this goal, a chemoprevention study was conducted to evaluate the activity of resveratrol (Res), a phytoalexin, as an inhibitor of colon carcinogenesis. Wistar male rats were divided into six groups, group 1 were control rats, group 2 were control rats that received Res (8 mg/kg body wt p.o. everyday), rats in groups 3–6 were treated weekly with 1,2-dimethylhydrazine (DMH, 20 mg/kg body wt, s.c. \(\times 15\) times). In addition, groups 4, 5 and 6 received Res as in group 2. Modifying effects were assessed using aberrant crypt foci (ACF) and the extent of histopathological lesions as end point markers. At the end of 30 weeks, Res markedly reduced tumor incidence, the degree of histological lesions and also the size of tumors significantly \((P < 0.05)\) as compared with the rats treated with unsupplemented DMH. The number of ACF consisting of more than six aberrant crypts per rat was observed in group 6 (6.2 \(\pm 1.4\)), group 5 (7.7 \(\pm 1.0\)) and group 4 (8.2 \(\pm 1.4\)) which were significantly lower than that of group 3 (22.3 \(\pm 2.4\)) \((P < 0.05)\). The most pronounced inhibition of ACF development was noted in rats fed Res for the entire period and also during the post-initiation period. Also, Res administration lowered the number of argyrophilic nucleolar organizing region-associated proteins (AgNORs) per nucleus in non-lesional colonic crypts, which reflects the cell proliferation activity. Oxidative imbalance in DMH-treatment was significantly \((P < 0.01)\) modulated on Res supplementation as indicated by optimal concentration of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH). The results of our study suggest Res to be an effective chemopreventive agent, which suppresses DMH-induced colon carcinogenesis at various stages.

**Introduction**

Colorectal cancer is the second most frequent cause of cancer mortality in the United States and the third most common cancer worldwide (1). Colon carcinogenesis is now established to be a multistep process characterized by the accumulation of genetic alterations involving a variety of oncogenes and tumor suppressor genes. Diet is considered as one of the major factors accounting for the variability in cancer incidence and mortality at these sites (2). Plant phytochemicals (biologically active, non-nutritive compounds) are receiving considerable attention for their potential role in reducing cancer risk.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) (Res), a phytoalexin produced by more than 70 different plant species, can be found in high to moderate quantities in various foods including grapes, peanuts and wine (3). More recently, there has been increasing interest in Res because of reports on its anticarcinogenic properties as evaluated by its effects on the major stages (initiation, promotion and progression) of malignant transformation (4). Figure 1 shows the structure of Res.

Intense interest in Res as a cancer preventive agent has increased for five main reasons: (i) wide range of availability in plants (5,6), (ii) wide range of target organs in rodent carcinogenic experiments (7,8), (iii) growth inhibition of various human cancer cell lines (9,10), (iv) wide distribution of \(^{14}\text{C}\) Res in various organs of rats (11) and (v) no adverse effects with Res even in higher doses in rats (12,13). Recent reports have demonstrated that oral administration of Res initiates a dramatic decrease in the number of tumors in the small intestine and also completely suppresses tumor formation in the colon of Min mice (14). A wide body of data indicates that Res has many important biological properties including inhibition of lipid peroxidation, free radical scavenging and anti-inflammatory activity (15).

Aberrant crypt foci (ACF) are putative neoplastic lesions that are induced in the colon of carcinogen-treated rodents and are present in humans with a high risk of colon cancer development (16,17). ACF are characterized by elevated crypts, thicker epithelial cell lining and increased pericryptal zone relative to normal crypts (18). Prevention of 1,2-dimethylhydrazine (DMH)-induced ACF has been used as a bioassay to assess the chemopreventive activity (19). Cell

**Abbreviations:** ACF, aberrant crypt foci; AgNOR, argyrophilic nucleolar organizing region; CAT, catalase; CMC, carboxymethylcellulose; DMH, 1,2-dimethylhydrazine; GSH, reduced glutathione; NOR, nucleolar organizing region; PCNA, proliferating cell nuclear antigen; Res, resveratrol; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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Fig. 1. Structure of Resveratrol.

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proliferation plays an important role in multistage carcinogenesis with multiple genetic changes. Therefore, control of cell proliferation is important for cancer prevention (20). In addition to DNA precursor uptake (bromodeoxyuridine) and immunohistochemical analysis of cell cycle proteins, proliferating cell nuclear antigen (PCNA), and the number of argyrophilic nucleolar organizer region associated proteins (AgNORs) are used to evaluate cell proliferation in various organs (21) including colonic mucosa.

Mechanisms by which certain carcinogens cause carcinogenesis is believed to be mediated by free radicals. Hydrazines and its derivatives, DMH and isoniazid, which can produce active oxygen species (22), have been shown to induce DNA damage process and carcinogenesis which can be abolished by free radical scavengers, also provide indirect evidence of the involvement of radicals in carcinogenesis (23). Antioxidant defense system, a key intracellular component capable of protecting cellular constituents from the attack of peroxides and free radicals, thus alters the events in the process of tumorigenesis (24). Much of the evidence has come from the fact that antioxidants scavenge free radicals directly or interfere with the generation of free radical-mediated events and inhibit the neoplastic process (22).

The present study was designed to investigate the effect of Res on DMH-induced colon carcinogenesis in Wistar rats with respect to ACF formation, antioxidant status and expression of cell proliferation biomarkers such as the number of AgNORs per nucleus in the colonic mucosa and to specifically investigate the effectiveness of this agent, when administered during the initiation, post-initiation and also throughout the entire period of the study. Successful treatment of neoplastic disease largely depends on inflicting the maximum damage on tumor stem cells and minimum damage on normal tissue stem cells. If drugs are administered continuously, there is no opportunity for stem cell recovery and hence differences between normal and tumor tissue will not be apparent. This study design allows us to elucidate the role of Res in inhibiting the formation, progression, and growth of premalignant and malignant colonic lesions.

Materials and methods

Animals and animal husbandry

Male adult Wistar rats of body weight 120–150 g were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University, Annamalainagar, and were acclimatized to the control diet for 1 week. Animals were maintained as per the principles and guidelines of the Ethics Committee of Animal Care of Annamalai University in accordance with the Indian National Law on animal care and use (Reg. No. 160/1999/CPCSEA). The animals were housed four per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk in a specific-pathogen free animal room under controlled conditions of a 12 h light / 12 h dark cycle, with temperature of 22 ± 1°C and relative humidity of 50 ± 10% till the end of the experimental period.

Chemicals

1,2-Dimethylhydrazine, methylene blue, hematoxylin, eosin and trans-resveratrol were purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals used were of analytical grade and obtained from Hi-Media Laboratories, Mumbai.

Carcinogen administration

All the animals in groups 3–6 received 20 mg/kg body wt DMH injections once a week subcutaneously for the first 15 weeks. Prior to subcutaneous injection, DMH was dissolved in 1 mM EDTA, the pH adjusted to 6.5 with 1 mM NaOH and used immediately.

Chemopreventive agent administration

Due to its low solubility in water, Res (8 mg/kg body wt) was suspended in carboxymethylcellulose (CMC) and given orally using an intragastric tube. The dose was adjusted according to the animal weight to ensure a constant dose. Dose preparation and administration were performed in dim light to avoid trans-resveratrol getting isomerized to the cis form.

Experimental design

Rats were assayed into experimental groups [16 per group; 8 for ACF analysis and 8 for AgNORs and hematoxylin and eosin (H&E) staining], using a randomization process designed to ensure comparable initial body weights in all study protocols. Animals were clinically examined every week and the body weight measured throughout the experimental period of 30 weeks. The experimental protocol is clearly represented in Figure 2.

Group 1 (Control). Rats received basal diet along with 100 mg/kg body wt CMC dissolved in water, throughout the experiment.

Group 2 (Control + Res). Rats received basal diet + 8 mg/kg body wt Res suspended in CMC, p.o. everyday throughout the experiment.

Group 3 (DMH). Rats were administered 20 mg/kg body wt DMH s.c. once a week for first 15 weeks.

Group 4 DMH + Res (l). The animals were administered DMH and also fed Res every day for the first 15 weeks starting 2 weeks before carcinogen treatment (Initiation-1).

Group 5 DMH + Res (PI). The animals were administered DMH and also Res 2 days after the last injection of the carcinogen and continued till the end of the experiment (Post-initiation-PI).

Group 6 DMH + Res (EP). The animals were administered DMH and Res starting from the day of carcinogen treatment and continued till the end of the entire experimental period of 30 weeks (Entire period-EP).

Necropsy and tissue preparation

All animals were anaesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg body wt), killed by cervical dislocation and the abdominal cavities were examined to reveal any macroscopic changes.

Preparation for ACF counting

The colon was processed as follows for the determination of ACF (25). The entire colon (from cecum to anus) was removed and washed thoroughly with 0.9% NaCl, cut longitudinally, laid flat on a polystyrene board, and fixed with 10% buffered formaldehyde solution overnight. The colon was then stained with 0.2% methylene blue for 3–5 min in saline in order to identify ACF, formed by one or more aberrant crypts, which are easily visualized on a background of normal crypts since aberrant crypts have larger, often elongated openings and a thicker lining of epithelial cells compared with normal crypts. For topographic assessment of the colon, mucosal ACF was counted using a light microscope (40X). The ACF were classified as small (1–3); medium (4–6); or large (>6) by the number of crypts per foci. The total number of ACF/rat was calculated as the sum of the small, medium and large ACF. To obtain additional information about the morphology of ACF and to confirm their geographical position within the colonic epithelium, sections containing ACF were marked and embedded in paraffin, sectioned and stained with H&E.

Tumor analysis and measurement of AgNORs number in colonic mucosa

Naked eye appearance of any tumors, including measurement of two greatest diameters using the formula for surface area of an ellipse (π × r1 × r2) with Vernier callipers were recorded to indicate the size of the tumor and then fixed in 10% buffered formalin. Transverse blocks of all tumors were taken and processed through to paraffin wax, fixed tumors were cut into serial sections of 3 μm wide strips and stained with H&E for histological examination. Staining for AgNORs was done to estimate cell proliferation. The AgNOR count of the mucosal epithelium of the colon in each group was determined. The colon was removed and divided into three equal portions, namely the upper, middle and lower colon and were used for analysis.

AgNOR staining

AgNOR staining was carried out by the method of Murray et al. (26). Silver colloid solution was made up with 2 g/dl gelatin in 1% aqueous formic acid. This solution was mixed with twice its volume of 50 g/dl aqueous silver nitrate solution. Sections were exposed to the staining solution for 60 min at room temperature. The silver colloid solution was allowed to dry at room temperature in a desiccator. All sections were counter-stained with 1% neutral red for 2 min. The slides were washed in tap water for 10 min, dehydrated through graded alcohol, cleared in xylene, and mounted using DPX.

For determination of the AgNOR count, the cell nuclei of 25 well-oriented crypts, in which the base, lumen and top of the crypts could be seen, were used. AgNORs were visualized as distinct silver positive black dots and clusters. AgNORs were counted on AgNOR-stained sections under a microscope at a magnification of 400X. Two hundred nuclei were assessed and the mean number of dots and clusters per nuclei were calculated separately for each
All sections were examined without knowledge of their group of origin.

**Histological definitions**

Tumors were macroscopically sessile or pedunculated in shape. Adenomas were defined histologically as lesions in which neoplastic cells were confined to the mucosal layer. Adenomas were further classified into three types according to the grade of atypia, while adenocarcinomas were those in which neoplastic cells had penetrated the muscularis mucosa to invade the submucosa or deeper layers. As previously reported (27), adenocarcinomas were further classified as well-differentiated or mucinous carcinomas. In well-differentiated carcinomas, tumor cells were found in acinar clusters mimicking the glandular crypts of normal intestinal mucosa. In mucinous carcinomas, mucin secretion was active, resulting in mucinous nodules that contained large amounts of extracellular mucin with only a few isolated groups of tumor cells.

**Tissue analysis**

The rat colons were placed on an ice-cold glass plate, cut open length wise, and scraped gently with an ice-cold microscope slide to collect the mucosa. Liver sections and colonic mucosal scrapings were homogenized in ice-cold phosphate buffer (pH 7.0, 0.01 M) with a Teflon pestle in a glass tube. Lipid peroxidation in fresh whole tissue homogenate was assessed by measuring thiobarbituric acid reactive substances (TBARS) by the method of Buege and Aust (28). The activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were assessed in the liver and mucosal homogenates as described previously (29,30). Liver and colonic reduced glutathione (GSH) levels were determined by using the method of Boyne et al. (31). Protein content was determined by the method of Lowry et al. (32).

**Statistical analysis**

Values are expressed as mean ± SD of 8 animals in each group. Data within and between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT). A value of $P < 0.05$ was considered statistically significant. Analysis was performed using SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA).

**Results**

Effect of DMH and Res on change in body weight and growth rate in control and experimental animals is shown in Figure 3. The change in body weight of DMH-treated animals maintained on the control diet showed a decrease as compared with groups 4, 5 and 6. In terms of any toxic effects of Res administration as monitored by change in body weight of control animals treated with Res, no noticeable differences were observed.

Growth rate was calculated as the difference between the final weight and the initial weight divided by the total number of days, i.e. 210. There was a significant ($P < 0.05$) increase in the growth rate on Res supplementation to DMH-treated rats (groups 4, 5 and 6) as compared to unsupplemented DMH-treated rats.

Effect of DMH and Res on tumor incidence, histopathological type, and depth of involvement of colonic tumors is represented in Tables I, and II. Representative photomicrographs
are shown in Figure 4, and the results are summarized in Table III. The colon tumor incidence (number of rats with tumors), multiplicity (number of tumors/rat) of the colon tumors in the treatment groups is summarized in Table I. No tumors were observed in control animals (group 1) and control rats fed Res (group 2). Macroscopically, most tumor development was observed in the large intestine (especially in the distal colon). No neoplasm was found in any of the other organs examined from rats in groups 3–6. Colon tumors observed in groups 3–6 were sessile or pedunculated and histologically they were adenomas and/or adenocarcinomas, with a higher incidence of adenocarcinoma. The frequency of large intestinal adenocarcinomas in group 3 was 63%, which was significantly higher ($P < 0.05$) than that of groups 4, 5 and 6 (58, 44 and 0% respectively). Significant inhibition occurred in the multiplicity of colon tumors and a significant reduction was observed in the inflammatory cell infiltration, both in the mucosal and submucosal layers of DMH-treated rats fed Res (groups 4, 5 and 6) as compared with unsupplemented DMH-treated rats (group 3), was shown in Table III. There was also a significant reduction in size and number of tumors (Table I) in the three treatment regimens, though only post-initiation and entire period treatments were statistically significant ($P < 0.05$).

Effect of DMH and Res on ACF formation is shown in Table IV. The rats treated with DMH (groups 3–6) showed 100% ACF incidence, in contrast to the complete lack of such lesions in control groups (groups 1 and 2). The number of ACF consisting of more than six aberrant crypts per rat in group 6 (6.2 ± 1.4), group 5 (7.7 ± 1.0) and in group 4 (8.2 ± 1.4) were significantly lower than that of group 3 (22.3 ± 2.4) animals ($P < 0.05$).

Effect of DMH and Res on the number of AgNORs in the colonic crypts is shown in Table V. The mean number of AgNORs/nucleus in group 3 was the greatest, and that in group 6 was the smallest of all groups. The number of AgNORs/nucleus was significantly decreased in all the three groups of rats treated with Res (initiation, post-initiation and entire period) as compared to unsupplemented DMH-treated rats (group 3). The effect of Res in reducing cell proliferation was more pronounced in groups 5 and 6.

Effect of DMH and Res on tissue TBARS is shown in Figure 5. Liver TBARS was significantly elevated ($P < 0.05$), whereas colonic mucosal TBARS was significantly reduced ($P < 0.05$) in the DMH-treated rats (group 3) at the end of 30 weeks as compared with the control rats (group 1). Res supplementation during the initiation (group 4) and post-initiation (group 5) stages of carcinogenesis did not have a significant effect on either the colonic mucosal or hepatic TBARS (except group 5) as compared with the unsupplemented DMH-treated rats (group 3). On the other hand supplementing Res throughout the period of the study (group 6) significantly elevated colonic mucosal TBARS ($P < 0.01$) and significantly lowered liver TBARS ($P < 0.01$) as compared with the rats (group 3) treated with DMH alone.

Effect of DMH and Res on tissue antioxidants is shown in Table VI. SOD, CAT, and GSH activities/levels were significantly decreased ($P < 0.05$) in liver and colonic mucosa of DMH treated rats (group 3) as compared to the control rats (group 1). Res supplementation during the initiation (group 4) and post-initiation (group 5) stages of carcinogenesis did not have a significant effect on the colonic and liver SOD, CAT and GSH levels as compared with the unsupplemented DMH-treated rats (group 3). But supplementing Res during the post-initiation stage as well as through the entire experimental period significantly elevated the liver and colonic SOD, CAT ($P < 0.05$) and GSH levels ($P < 0.05$) as compared with the rats (group 3) exposed to DMH alone. The effect of Res in restoring tissue antioxidant status was more pronounced in group 6 ($P < 0.01$) as compared with rats treated with DMH alone.
Discussion

Human colon carcinogenesis is usually associated with a combined action of various kinds of xenobiotics and autobiotic carcinogenic factors (33). The use of antioxidants as inhibitors of tumorigenesis has received much attention. The precise mechanism of action of these compounds is presently unknown although proposals include, scavenging of ultimate carcinogenic metabolites and alteration of enzyme systems responsible for the activation of procarcinogens (34). In the present study, we demonstrate that Res has a potent inhibitory activity against DMH-induced tumor formation in the rat colon when Res was given to rats at a dose of 8 mg/kg body wt in three different regimens (initiation, post-initiation and entire period) and the body weight gain was significantly improved as compared with rats treated with unsupplemented DMH. This may be due to the reduced DMH carcinogenicity and consequent tumor burden. DMH is a colon-specific carcinogen (35), which is metabolically activated in the liver and then delivered to the colon via the blood stream or via bile as glucuronide conjugates. After further activation, it methylates DNA mainly at the N1 and O6-positions of guanine (36). DNA adduct formation is considered to be the initiating step in the formation of tumorigenesis (37).

Res when given as post-initiation regimen or through the entire period of the study, the expression of larger ACF in the distal colon was reduced to a great extent, as compared with the unsupplemented DMH-treated group. Larger ACF (six or more aberrant crypts per focus) are considered more likely to progress into tumors (38) and in this study Res feeding had a significant inverse influence on larger ACF formation in the distal colon. ACF may regress or become indolent, while some develop into microadenomas and some into adenomas and with time and further mutations, some of these give birth to adenocarcinomas (39). A previous study has shown that the administration of Res inhibits tumorigenesis and modulates host-defense related gene expression (14). Our results also suggest that Res inhibits the growth of colonic ACF and suppresses progression of preneoplasia to malignant neoplasia. Although the mechanisms involved in the protective effects against ACF formation are not clearly understood, the inhibitory action of Res could be explained by its putative antioxidant activity. In this context, other chemopreventive agents with antioxidant properties have been found to inhibit DMH and azoxymethane-induced colon carcinogenesis and DNA damage in an animal model (40).

In the current study, we have also histologically characterized the tumor type in the colon to determine if the protective effect occurred equally for benign and invasive adenocarcinoma, and evaluated if any regional differences existed. It was found that the incidence of invasive adenocarcinoma was significantly lower in all the three Res fed groups. The inhibitory effect of Res on colon carcinogenesis induced by DMH in rats is evidenced by the decreased incidence and multiplicity of colonic tumors. The comparison of the three regimens of treatment with Res showed that the inhibitory effect occurred
Table III. Effect of DMH and Res on histological appearance of colonic tissue in cancer and treatment groups

<table>
<thead>
<tr>
<th>Macroscopy</th>
<th>DMH</th>
<th>DMH + Res</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean tumor size (mm²)</td>
<td>26.8 ± 23.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.2 ± 16.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nature</td>
<td>Sessile</td>
<td>Pendunculated</td>
</tr>
<tr>
<td>Margin</td>
<td>Well defined</td>
<td>Defined</td>
</tr>
<tr>
<td>Microscopy</td>
<td></td>
<td>III-defined</td>
</tr>
<tr>
<td>Mucosal layer</td>
<td></td>
<td>III-defined</td>
</tr>
<tr>
<td>Infiltration of inflammatory into the mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosal crypt architecture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Crypt branching</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Crypt enlargement-transitional mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crypt epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) Mucin production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d) Epithelial cell numbers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(e) Cell morphology</td>
<td>Degenerated</td>
<td></td>
</tr>
<tr>
<td>1. Atypia with nuclear hyperchromatism</td>
<td>Marked</td>
<td>Less severe</td>
</tr>
<tr>
<td>2. Pleomorphism</td>
<td>Marked pseudoatratification</td>
<td>Less severe</td>
</tr>
<tr>
<td>3. Stratification of nuclei</td>
<td>Numerous</td>
<td>Present</td>
</tr>
<tr>
<td>4. Mitotic figures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltration of inflammatory cells in the submucosa</td>
<td>Marked</td>
<td>Less severe</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>Marked</td>
<td>Less severe</td>
</tr>
<tr>
<td>(f) Fibrosis</td>
<td>Marked</td>
<td></td>
</tr>
<tr>
<td>(g) Vascular granularity and vascular congestion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Values are expressed as mean ± SD.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table IV. Effect of DMH and Res on the number of ACF

<table>
<thead>
<tr>
<th>Groups</th>
<th>ACF/ colon</th>
<th>DMH</th>
<th>DMH + Res</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Medium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Large&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH</td>
<td>35.6 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.4 ± 5.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.3 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH + Res (I)</td>
<td>25.7 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.5 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.2 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH + Res (PI)</td>
<td>17.6 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.1 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH + Res (EP)</td>
<td>12.1 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.

Table V. Effect of DMH and Res on the number of AgNORs in crypt cell nuclei

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats examined</th>
<th>No. of crypts examined</th>
<th>Mean cells/ crypt column</th>
<th>No. of AgNORs/ nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>25</td>
<td>26.8 ± 4.3</td>
<td>2.7 ± 5.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH</td>
<td>8</td>
<td>25</td>
<td>21.6 ± 4.5</td>
<td>8.2 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH + Res (I)</td>
<td>8</td>
<td>25</td>
<td>21.6 ± 5.3</td>
<td>1.4 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH + Res (PI)</td>
<td>8</td>
<td>25</td>
<td>26.0 ± 5.3</td>
<td>1.4 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH + Res (EP)</td>
<td>8</td>
<td>25</td>
<td>25.1 ± 3.1</td>
<td>1.3 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.

Mainly at the promotion and progression stages of DMH-induced colon carcinogenesis. Importance of the interactions between immune cells and neoplastic cells in influencing the pathogenesis of colon cancer is well documented (41). A number of experiments (42) indicate that the NF-κB transcription factor is involved in the development or progression of cancers. Res, probably arrests the cell division cycle at S/G2 phase transition and suppresses the activity of NF-κB, which is associated with proliferation, differentiation and cell death (43,44). Thus, Res supplementation may down-regulate NF-κB mediated immune responses, thereby acting as an immunomodulatory agent. It could be suggested that the immunomodulatory effect of Res also plays a role in its anticarcinogenic potential (45).

Nucleolar organizer regions (NOR) are loops of DNA that contain ribosomal RNA genes. These genes are transcribed by RNA polymerase I and ultimately result in direct ribosomal formation and protein synthesis. NOR-associated acidic proteins are related to sites of rRNA transcription that can be demonstrated in the interphase nucleus in histological sections by silver-staining. Moreover, the products can be visualized as black dots, which are referred to as AgNORs. It has been suggested that the number of AgNOR dots in a nucleus may reflect the status of cell activation and therefore is a useful index to assess cell proliferation (46). Colonic epithelial cell proliferation changes are considered to be indicators of increased risk of colon cancer (47). Abnormal cellular proliferation is one of the important mechanisms in carcinogenesis (48).
Res down-regulates the expression of cyclins D1 and D2, which are directly involved in cell cycle progression (49). They are generally stimulated during malignancy (50) and repressed by anticancerous phytochemicals (51). Res is known to reduce significantly the expression of transcription factors, including the DP-1 transcription factor, which is involved in the control of cell proliferation (52). Res have also been shown to possess anti proliferative, cell cycle arresting properties in vitro on several cancer cell lines (53). Our data indicating reduced number of AgNORs/nucleus suggest that the mechanism of reduction of ACF number by Res may be due to decreased cell proliferation.

Oxidative stress occurs when the production of free radicals increases, scavenging of free radicals or repair of oxidatively damaged macromolecules decreases, or both. One of the plausible ways to prevent reactive oxygen species mediated cellular injury is to augment or fortify endogenous defense capacity against oxidative stress through dietary or pharmacological intake of antioxidants (54). At 30 weeks, consistently increased lipid peroxidation was observed in the liver of DMH-treated rats. Liver antioxidant enzymes were also assessed to determine whether impaired activity of the antioxidants might have contributed to oxidative stress. Liver SOD, CAT and GSH levels were significantly lower in DMH-treated rats as compared with the control and thus may have contributed to the increased liver lipid peroxidation.

In the present study Res supplementation significantly decreased hepatic TBARS associated with increased activities of SOD, CAT and GSH levels. This antioxidant property of Res may be responsible for protecting the cells against the oxidative stress, possibly by increasing the endogenous defensive capacity of the liver to combat oxidative stress induced by DMH. It may be noted, however that Res treatment for the entire period of the study was more effective than the other treatment regimens. Res is an electron-rich unsaturated amphiphatic molecule containing one resorcinol and one phenol moiety linked by an ethylene group capable of scavenging lipid hydroperoxyl, hydroxyl and superoxide anion radicals. The phenolic group of Res is also involved in this activity (55).

Unlike liver lipid peroxidation, colonic TBARS were not increased on treatment with DMH. Previous studies proved that rats administered DMH for a long period had decreased colonic lipid peroxidation as compared with the control rat tissue (56). Our data also demonstrate reduced lipid peroxidation in DMH treated rats. It is generally believed that there is an inverse relationship between the concentration of lipid peroxides and the rate of cell proliferation (57) and differentiation (58). In our study Res supplementation to DMH-treated rats had higher levels of TBARS. This finding suggests Res can protect cells from loss of their oxidative capacity due to the administration of the procarcinogen DMH. The enzymes SOD, CAT and GSH play a key role in the cellular defense against free radical damage (57). Significant increase in SOD, CAT activities with concomitant GSH replenishment on Res treatment, could be important in inhibiting carcinogenesis induced by DMH. These observations are corroborated by earlier reports on the antioxidant activity of Res in murine breast cancer (59) and two-stage model of skin cancer (4).

From a practical point of view, those inhibitors, which act during promotion and progression stages, are more likely to be useful for the prevention of human cancer. Since initiation is rapid, especially in the colon, the duration of treatment with inhibitors is crucial. Promotion, on the other hand, is considered as reversible and generally a slow process influencing the proliferation of initiated cells. Therefore, the possibility of blocking this process is much greater. Thus, agents that inhibit the promotion stage of carcinogenesis, or both initiation and promotion events could offer one of the most effective methods of cancer prevention.

**Table VI.** Effect of DMH and Res on tissue SOD, CAT activities and GSH levels

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>SOD Enzyme required for 50% inhibition of NBT reduction/min/mg protein</th>
<th>CAT μmol H2O2 utilized/min/mg protein</th>
<th>GSH mmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Colon</td>
<td>Liver</td>
</tr>
<tr>
<td>Control</td>
<td>7.43 ± 0.63^a</td>
<td>3.80 ± 0.33^a</td>
<td>43.11 ± 4.19^a</td>
</tr>
<tr>
<td>Control + Res</td>
<td>7.31 ± 0.73^a</td>
<td>3.70 ± 0.25^a</td>
<td>45.21 ± 4.21^a</td>
</tr>
<tr>
<td>DMH</td>
<td>3.20 ± 0.39^b</td>
<td>2.30 ± 0.19^b</td>
<td>23.99 ± 2.14^b</td>
</tr>
<tr>
<td>DMH + Res (I)</td>
<td>3.36 ± 0.19^b</td>
<td>2.40 ± 0.16^b</td>
<td>25.30 ± 2.18^b</td>
</tr>
<tr>
<td>DMH + Res (PI)</td>
<td>4.32 ± 0.25^bc</td>
<td>2.51 ± 0.18^bc</td>
<td>29.15 ± 2.26^bc</td>
</tr>
<tr>
<td>DMH + Res (EP)</td>
<td>6.13 ± 0.35^d</td>
<td>3.16 ± 0.18^d</td>
<td>39.53 ± 3.71^c</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of 6 rats.

Values not sharing common superscript (a–d) are significant with each other at *P < 0.05; *P < 0.01 versus DMH (ANOVA followed by DMRT).
Cumulatively, our results suggest that Res can protect colonic tissues against DMH-induced colon carcinogenesis in rats. This protective effect of Res against rat colon tumorigenesis was more striking when Res was supplemented in entire period of the study. This is evident by the reduced colonic tumor incidence, rate, size and multiplicity coupled with decrease in the number of ACF. The chemopreventive effect was more significant when Res was administered during the promotion and progression stages of carcinogenesis. Thus Res might have practical applications as a chemopreventive agent, providing a scientific basis against human colon carcinogenesis.

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References

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