DOSE DEPENDENT EFFECT OF GERANIOL ON LIPID PEROXIDATION IN 1,2-DIMETHYL HYDRAZINE INDUCE RAT COLON CARCINOGENESIS IN MALE WISTAR RATS

Sudha Mani and Namasivayam Nalini*

Department of Biochemistry, Sri Akilandeswari Women’s College, Vandavasi - 604 408, Tamilnadu, India.

Abstract

Colorectal cancer is the third most common internal malignancy in the Western society. Currently oxidative stress has been increasingly postulated as a major contributor or carcinogenesis. Our current study is an effort to identify a potent chemopreventive agent against colon cancer. Here, we have investigated the efficacy of geraniol on tissue oxidative stress, antioxidant defence system and colonic histoarchitecture in colon cancer bearing male wister rats. Group 1 rats received modified pellet diet and served as control. Group 2 received modified pellet diet along with geraniol (200 mg kg body weight, p.o, everyday). Rats in group 3, 4, 5 and 6 were treated with DMH (20 mg kg body weight) once a week for the first 4 weeks. In addition geraniol was supplemented at the dose of 50, 100 and 200 mg/kg body weight every day orally for the entire period of 16 weeks group 4 – 6) respectively. DMH treatment showed increased levels of lipid peroxidation in the plasma and hepatic tissues and decreased levels in the colonic tissue. Supplementation with geraniol at three different doses (50, 100, 200 mg/kg body weight) significantly restored lipid peroxidation by products as compared to the unsupplemented DMH treated groups. Thus, our results indicate that geraniol markedly inhibits DMH induced colon carcinogenesis. The chemopreventive efficacy of geraniol was more pronounced at the dose of 100 mg/kg body weight.

Key words: 1,2-dimethyl hydrazine, Lipid peroxidation Geraniol, Chemopreventive efficacy and Histoarchitecture.

1. Introduction

Cancers of the large and small intestine are major contributors to worldwide cancer morbidity and mortality. Colon cancer is thought to arise from the accumulation of mutations in a single epithelial cell of the colon and rectum (Fearon and Vogelstein, 1990). Relentless cell proliferation and rapid invasion into surrounding tissues are fundamental characteristics of malignant tumour cells (Herlyn and Malkowicz, 1991).

*Corresponding author: Namasivayam Nalini
E.mail: nalininam@yahoo.com
countries, but is increasing with increasing migration of rural population to the cities, increase in life expectancy and changes in lifestyle (Srikhande et al., 2007). Out of 3.5 million cancer cases in India, 35,000 suffer from colon cancer (Srikhande et al., 2007).

Colon cancer is frequently a pathological consequence of persistent oxidative stress, leading to DNA damage and mutations in cancer–related genes, a cycle of cell death and regeneration (Bartsch and Nair, 2002), where cellular overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated. Antioxidant defence system, a key intracellular component capable of protecting cellular constituents from the attack of peroxides and free radicals, alters the events in the process of tumourigenesis.

Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is an acyclic monoterpene alcohol. It is an important constituent of essential oil of ginger, lemon or lime, lavender, nutmeg, orange, rose etc. Geraniol is used as a flavouring agent and also in perfumes due to its rose like fragrance. Geraniol has a variety of pharmacological effects such as antimicrobial effect, antioxidant, anti-inflammatory and anti-carcinogenic effects (Carnesechi et al., 2001).

2. Material and methods

Chemicals

Geraniol and 1,2-dimethylhydrazine were purchased from Sigma Chemical Co. (USA). All other chemicals and reagents used were of analytical grade.

Animals

Four-week old male Wistar rats, weighing approximately 150 g, were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMCH). They were kept in a plastic cages with bedding and maintained under controller conditions of temperature (22 ± 2 °C), humidity (55 ± 10 %), 12 hrs light/dark cycle and provided with modified pelleted diet (Table - 1) containing 20 % fat (peanut oil) and tap water Ab libitum. Animal handling and the experimental design were approved by the Animal Care and Use Committee of Annamalai University (Reg. No. 190/2007/CPCSEA/685).

Table - 1: Composition of the modified pelleted diet

<table>
<thead>
<tr>
<th></th>
<th>Commercial pelleted diet 84.2 %</th>
<th>Peanut oil 15.8 %</th>
<th>Total 100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>17.7</td>
<td>-</td>
<td>17.7</td>
</tr>
<tr>
<td>Fat</td>
<td>4.2</td>
<td>15.8</td>
<td>20.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>50.5</td>
<td>-</td>
<td>50.5</td>
</tr>
<tr>
<td>Fibre</td>
<td>3.4</td>
<td>-</td>
<td>3.4</td>
</tr>
<tr>
<td>Minerals</td>
<td>6.7</td>
<td>-</td>
<td>6.7</td>
</tr>
<tr>
<td>Vitamins</td>
<td>1.7</td>
<td>-</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Tumor induction

DMH was dissolved in 1 mM EDTA just prior to use and the pH adjusted to 6.5 with 1 mM NaOH to ensure the stability of the carcinogen. The rats were given subcutaneous injections of DMH at a dose of 20 mg/kg bodyweight for 4 consecutive weeks.

Formulation of geraniol

Geraniol was suspended in corn oil just before use and was administered everyday orally at the doses of 50mg, 100 mg or 200 mg/kg bodyweight throughout the experimental period of 16 weeks.

Experimental design

Rats were randomly distributed into six groups of 8 rats each, as follows: group 1 received modified pelleted diet and served as control; group 2 received modified pelleted diet with geraniol (50 mg /kg body weight) everyday orally by intragastric intubation throughout the experimental period; groups 3-6 received modified pelleted diet with
DMH injections (20 mg /kg body weight) once a week, subcutaneously for the first four weeks. In addition, groups 4-6 received 50, 100 or 200 mg /kg body weight of geraniol in 0.1 % of corn oil orally by intragastric intubation every day, for the total period of 16 weeks, respectively. Schematic representation of the experimental design is shown in Figure - 2. Body weights of rats were recorded initially, at weekly intervals and also at the end of the experiment.

Figure - 2: Effect of geraniol and DMH on body weight

Lipid peroxidation

Lipid peroxidation as indicated by thiobarbituric acid reactive substances (TBARS) was measured using the method of Ohkawa et al. (1979). The levels of conjugated dienes (CD) was assessed by the method of Rao and Recknagel (1968).

Histopathological changes

After sacrifice, the colons were macroscopically examined for the presence of tumours or other pathological lesions. Tissues with abnormal morphology were fixed in 10 % buffered formalin and embedded in paraffin blocks. Sections stained with hematoxylin and eosin used to confirm the presence and type of tumours by histopathological examination, which was performed by a pathologist unaware of the experimental codes.

Statistical analysis

The statistical significance of the data was determined using one-way analysis of variance (ANOVA) and the significant difference among treatment groups was evaluated by Duncan’s multiple range test (DMRT). The results were considered statistically significant at P < 0.05. All statistical analyses were made using SPSS 11.0 software package (SPSS, Tokyo, Japan).

3. Result

Effect of geraniol and DMH on Lipid peroxidation by products

The Table - 2 shows the effect of geraniol and DMH on the levels of lipid peroxidation byproducts in the tissues and plasma of control and experimental rats. The lipid peroxidation byproducts such as TBARS and CD levels were significantly decreased in the colonic tissues and significantly increased in the liver and plasma of DMH treated rats (Group 3) as compared to the control rats (Group 1). The levels of the lipid peroxidation by products were significantly reduced on supplementation with geraniol at the doses of 50 mg, 100 mg and 200 mg/kg b.wt. More pronounced effect was observed in the carcinogen exposed rats supplemented with 100 mg/kg b.wt of geraniol.
Table - 2: Effect of geraniol and DMH on tissue TBARS and CD of control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Control + Geraniol (200 mg/kg b.wt.)</th>
<th>DMH</th>
<th>DMH + Geraniol (50 mg/kg b.wt.)</th>
<th>DMH + Geraniol (100 mg/kg b.wt.)</th>
<th>DMH + Geraniol (200 mg/kg b.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS Plasma (µmoles/mL)</td>
<td>2.04±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.83±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.63±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.91±0.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.97±0.38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.61±0.54&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (mmol/mg tissue)</td>
<td>0.52±0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.49±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.58±0.05&lt;sup&gt;db&lt;/sup&gt;</td>
<td>0.45±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.59±0.05&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proximal colon (mmol/mg tissue)</td>
<td>0.44±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.42±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.35±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.40±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Distal colon</td>
<td>8.59±0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.32±0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.12±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.65±0.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.21±0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.44±0.71&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma (µmoles/mL)</td>
<td>0.81±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.83±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91±0.08&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.83±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.61±0.54&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (mmol/mg tissue)</td>
<td>54.06±53.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.04±5.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.60±7.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.42±6.97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50.97±4.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.69±0.54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proximal colon (mmol/mg tissue)</td>
<td>66.31±6.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.28±6.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>41.01±3.94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.12±5.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>64.77±6.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.26±5.60&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

Data are presented as means ± SD of eight rats in each group. (a-d) P<0.05, Values not sharing a common superscript letter are significantly different

**Effect of geraniol and DMH on liver and colon histology**

The Figure – 3 represents the photomicrographs of the hematoxylin and eosin staining of the liver sections of the control and experimental rats. Control rat shows normal liver section lobules and portal areas with central veins. Control + geraniol (200 mg/kg b.wt.) treated rat shows normal liver section with no changes in liver morphology. DMH (Group 3) treated rat liver shows dysplastic foci with nuclear hyperchromasia and enlargement. DMH + geraniol (50 mg/kg b.wt.) treated rat liver shows mild dysplastic changes. DMH + geraniol (100 mg/kg b.wt.) treated rat liver shows reduction in dysplastic areas with normal architecture. DMH + geraniol (200 mg/kg b.wt.) treated rat liver shows dysplastic cells with moderate nuclear hyperchromasia. The Figure - 4 represents the photomicrographs of the hematoxylin and eosin staining of the colon sections of the control and experimental rats. Control rat shows normal colon with many goblet cells. Control + geraniol (200 mg/kg b.wt.,) treated rat shows normal colon with reduction in goblet cells. DMH (Group 3) treated rat colon shows reduced goblet cells and glandular crowding. DMH + geraniol (200 mg/kg b.wt.,) treated rat colon shows normal mucin and epithelial stratification. DMH + geraniol (100 mg/kg b.wt.,) treated rat colon shows reduced mucin with normal epithelial lining. DMH + geraniol (200 mg/kg b.wt.,) treated rat colon shows normal mucin and epithelial stratification.
Figure - 3: Photomicrographs of hematoxylin and eosin stained sections of colon under 40X standard light magnification.

a) Control rats shows normal colon with many goblet cells, b) Control + geraniol treated rat shows normal colon with reduction in goblet cells, c) DMH treated rat shows colon with reduced goblet cells and glandular crowding, d) DMH + geraniol treated rat shows colon with reduced mucin and epithelial stratification, e) DMH + geraniol treated rat shows colon with reduced mucin and normal epithelial lining, f) DMH + geraniol treated rat shows colon with normal mucin and epithelial stratification.
Figure - 4: Photomicrographs of hematoxylin and eosin stained sections of liver under 40X standard light magnification.

Normal liver lobules and portal areas with central veins, b) Control + geraniol treated rat shows no changes in liver morphology, c) DMH treated rat liver shows dysplastic foci with nuclear hyperchromasia and enlargement. d) DMH + geraniol treated rat liver shows mild dysplastic changes, e) DMH + geraniol treated rat liver shows reduction in dysplastic areas with normal architecture, f) DMH + geraniol treated rat liver shows dysplastic cells with moderate nuclear hyperchromasia.

4. Discussion

The study clearly indicates that administration of geraniol in the presence of the procarcinogen DMH appreciably attenuates the alterations in the tissue lipid peroxidation and the enzymic and non-enzymic antioxidant status. The medium dose (100 mg/kg b.wt) of geraniol was more effective than the lower (50 mg/kg b.wt) and the higher doses (200 mg/kg b.wt).

Oxidative stress is usually implicated during all the stages of cancer development as
well as in the genesis of other diseases (Shijun et al., 2000). Specifically, evidences indicate the generation of ROS in various carcinogenic processes (Manju and Nalini, 2005). Cancer cells acquire particular characteristics that benefit their proliferation (Schmelz et al., 2000). DMH is a procarcinogen which requires metabolic activation to its active electrophilic carcinogenic form through a series of oxidative steps in the liver (No et al., 2007). Lipid peroxidation as evidenced by the increased levels of TBARS and CD were significantly enhanced in the liver of DMH-treated rats, which could be attributed to DMH-induced oxidative stress, and production of reactive oxygen metabolites. Moreover, the levels of TBARS and CD were significantly decreased in the colonic tissues of DMH-treated rats. In this context, previous studies have shown reduced rates of lipid peroxidation (LPO) in the tumour tissues of various types of cancers (Cheesman et al., 1986; Tanaka, 1997; Tanaka et al., 1998; Pillai et al., 1999).

Enhanced LPO is associated with depletion of the detoxifying antioxidant enzymes (SOD, CAT, GPx, GR) in the erythrocytes (RBC’s). These are the characteristic findings in malignant transformations (Ray et al., 2000). It is evident that in DMH-treated rats, the oxidant homeostasis is disturbed. The active carcinogenic electrophiles (diazonium ions) that are produced during DMH metabolism in the liver are released into the circulation eventually leading to LPO in the plasma and RBC’s (Bobek et al., 2000). Thus, LPO was found to be increased in the plasma and RBC’s of DMH-treated rats as compared to control rats. Earlier reports suggest that tumour cells produce substantial amounts of H$_2$O$_2$ that are released into circulation (Manju and Nalini, 2005; Szatrowski and Nathan, 1991). In addition superoxide (O$_2^-$) and hydroxyl radicals (OH) are also released into the circulation resulting in increased susceptibility of the plasma and RBC’s to LPO in DMH-treated rats. Moreover, accumulation of H$_2$O$_2$ results in the formation of hydroxyl radical (OH) and a highly toxic hypochlorous acid as a result in metabolism of H$_2$O$_2$ by circulatory neutrophil derived myeloperoxidase. Thus, H$_2$O$_2$ produced by tumour cells elicit oxidative stress in the RBCs.

We have previously reported decreased levels of LPO in the colon of DMH-treated rats (Sengottuvelan et al., 2006). Although, the changes in LPO status may reflect cell growth rate rather than the malignancy per se, important changes nevertheless occur at some earlier stage during the progression of normal cells to malignancy. Tumour cells tend to proliferate faster when the LPO levels are low. Thus, the decreased colonic LPO observed in DMH-treated rats could be due to increased proliferation and there is an inverse relationship between the levels of cellular LPO and rates of cell proliferation and the extent of differentiation. In this context Navarro et al. (1999) and Diplock et al. (1994) have suggested that highly proliferating dedifferentiated tumour cells have notably low levels of LPO products as compared to untreated control rats. In addition, the decreased LPO in the colon of DMH-treated rats may also be due to increased resistance and decreased susceptibility of the target organs to free radical attack. The restoration of LPO levels near those of the control rats on supplementation with geraniol may be due to its strong antioxidant property (Choi et al., 2000; Edris, 2007). In this context, geraniol is known to scavenge free radicals such as superoxide and hydroxyl radicals by virtue of its antioxidant property (Choi et al., 2000). Moreover, our findings correlate with the hypothesis that chemopreventive agents act as anticarcinogenic compounds by modulating carcinogen-induced circulatory oxidative stress (Balasenthil et al., 2001).

Histopathological analysis of the colon showed malignant glands on DMH treatment (group 3). Geraniol supplementation to DMH treated (group 5) rats, showed reduced number of malignant glands. This shows that geraniol is anticarcinogenic and effectively inhibits DMH-induced colon tumours. In summary, our results strongly provide the experimental evidence that geraniol supplementation at an appropriate dose of 100 mg/kg b.wt protects against LPO acts as a chemopreventive agent against DMH-induced colon tumorigenesis. Geraniol acts as a free
radical quencher by preserving the cellular antioxidant status and protects the cells from LPO. Further studies are in progress, to assess the mechanism of action exhibited by geraniol, which in future could be developed as a successful chemopreventive agent to treat colon cancer.

5. References
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