Chemopreventive potential of zinc in experimentally induced colon carcinogenesis

Vijayta Dani, Ajay Goel, K. Vaiphei, D.K. Dhawan

Abstract

The present study was performed to evaluate the efficacy of zinc treatment on colonic antioxidant defense system and histoarchitecture in 1,2-dimethylhydrazine- (DMH) induced colon carcinogenesis in male Sprague–Dawley rats. The rats were segregated into four groups viz., normal control, DMH treated, zinc treated, DMH + zinc treated. Colon carcinogenesis was induced through weekly subcutaneous injections of DMH (30 mg/kg body weight) for 16 weeks. Zinc (in the form of zinc sulphate) was supplemented to rats at a dose level of 227 mg/L in drinking water, ad libitum for the entire duration of the study. Increased tumor incidence, tumor size and number of aberrant crypt foci (ACF) were accompanied by a decrease in lipid peroxidation, glutathione-S-transferase, superoxide dismutase (SOD) and catalase. On the contrary, significantly increased levels of reduced glutathione (GSH) and glutathione reductase (GR) were observed in DMH treated rats. Administration of zinc to DMH treated rats significantly decreased the tumor incidence, tumor size and aberrant crypt foci number with simultaneous enhancement of lipid peroxidation, SOD, catalase and glutathione-S-transferase. Further, the levels of GSH and GR were also decreased following zinc supplementation to DMH treated rats. Well-differentiated signs of dysplasia were evident in colonic tissue sections by DMH administration alone. However, zinc treatment to DMH treated rats greatly restored normalcy in the colonic histoarchitecture, with no apparent signs of neoplasia. EDXRF studies revealed a significant decrease in tissue concentrations of zinc in the colon following DMH treatment, which upon zinc supplementation were recovered to near normal levels. In conclusion, the results of this study suggest that zinc has a positive beneficial effect against chemically induced colonic preneoplastic progression in rats induced by DMH.

Keywords: Dimethylhydrazine; Antioxidant status; Zinc; Colon cancer

1. Introduction

Colon cancer is one of the leading causes of cancer related deaths in both men and women in western countries, including the Unites States (Landis et al., 1999). It is frequently a pathological consequence of persistent oxidative stress, leading to DNA damage, mutations in cancer related genes, as well as epigenetic silencing of tumor suppressor genes (Goel et al., 2001, 2006; Bartsch and Nair, 2002; Boland et al., 2005). The end consequence of such a genomic instability is cellular overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative DNA
damage may participate in ROS-induced carcinogenesis (Breimer, 1990). Formation of hydroxylated bases of DNA is considered an even important event in chemical carcinogenesis (Breimer, 1990; Bartsch and Nair, 2002). This adduct formation interferes with normal cell growth by causing genetic mutations and altered normal gene transcription.

The public health impacts from colon cancer has spawned a growing interest in prevention trials and among these dietary micronutrients are viewed as promising agents in colon cancer prevention. Both epidemiological and experimental studies suggest that colon cancer is strongly influenced by nutritional factors, including the quantity and composition of dietary fat (Willett et al., 1990). Several investigators have over many years, conducted research on agents with potential chemopreventive properties and have elucidated their modes of action. Although a full explanation of the intricacies of the causes, development and control of colon cancer is awaiting further research, however, there is enough scope to explore the use of new agents as interventions at various stages of cancer for the better management of the patients suffering from this pathological condition.

Nowadays, some recent evidence has indicated role of zinc in carcinogenesis. Zinc has been ascribed vital roles in the metabolism and interaction of malignant cells (Schrauzer, 1977). Zinc replenishment has been shown to induce apoptosis in esophageal epithelial cells, thereby providing growth inhibition for the development of esophageal cancer (Fong et al., 2001). The zinc content of leukemia cells has also been found to be reduced and reports indicate that zinc deficiency does enhance the carcinogenic effects of nitroso-methylbenzylamine (Fong et al., 1978). It has also been shown that a frequent biochemical characteristic of prostate cancer is the marked decrease in zinc levels in the malignant cells, thus providing compelling evidence that the lost ability of the malignant cells to accumulate zinc is an important factor in the development and progression of prostate malignancy (Costello et al., 2004).

Keeping in mind these limitations of the existing literature, the present study was designed to explore the possibility of zinc being used as a measure of long term prophylactic therapy in delaying the compounding events, leading to the development of colon tumors. These aims were achieved by evaluating its role in the regulation of key enzymes involved with the oxidative stress mechanisms, as well as investigations on the histological alterations in the colon of rats subjected to 1,2-dimethylhydrazine (DMH) (an organo-specific carcinogen) treatment.

2. Materials and methods

2.1. Chemicals

1,2-Dimethylhydrazine (DMH), NADPH, GSH, NBT, DTNB, were procured from Sigma–Aldrich company (Delhi, India). Zinc sulphate was purchased from E. Merck.

2.2. Animals

Male Sparque–Dawley rats in the weight range of 120–150 g were procured from the Central Animal House, Panjab University, Chandigarh. The animals were housed in polypropylene cages under hygienic conditions in the departmental animal house. Before initiating the experiments, the animals were adapted to the laboratory conditions for a week. Necessary approvals were obtained from the Ministry of Social Justice and Empowerment for the use of experimental animals in this study.

2.3. Experimental design

Animals were segregated into four treatment groups. Animals in Group I served as normal controls and were given water and diet ad libitum. Rats in this group in addition were also administered with 1 mM EDTA–saline subcutaneously per week, which was used as the vehicle for the treatment of DMH treated animals. Animals in Group II were given a weekly subcutaneous injection of DMH at a dose level of 30 mg/kg body weight dissolved in 1 mM EDTA–normal saline (pH 6.5), for a total duration of 16 weeks (Soler et al., 1999). Group III animals were given zinc in the form of ZnSO₄·7H₂O in drinking water ad libitum at a dose level of 227 mg/L of drinking water (Goel et al., 2005). Animals in Group IV were given a combined treatment of DMH as well as zinc in a similar manner as was given to Group II and Group IV animals, respectively.

2.4. Colon tumor analysis

After the terminal sacrifice following 16 weeks of DMH treatment, colons were excised from the rats, blotted dry, cut open longitudinally and the inner surface was examined for the visible macroscopic lesions. Tumors were easily discernable in the inflamed sections of the colon. The number of tumors was noted for tumor incidence and multiplicity studies. Tumor size was also noted and the three main axes of each macroscopic tumor from rats were measured using a vernier caliper with 0.1 mm graduation.

2.5. Preparation for aberrant crypt foci (ACF) counting

The entire colon was removed and washed thoroughly with 0.9% NaCl, cut longitudinally 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 iden-
2.6. Body weight changes

A careful record of body weight changes of control, DMH and zinc treated animals was kept throughout the study. The animals were weighed at the beginning of the experiment and twice a week, and finally before sacrificing them. A daily record of food as well as water intake was also maintained throughout the study period.

2.7. Preparation of colon homogenate

The rats were sacrificed by giving adequate ether anesthesia and the colons were removed immediately and washed with ice-chilled saline. The colons were weighed and a small portion was immersion fixed in 10% phosphate buffered formalin for histological examination at the light microscopic level.

Ten percent colon homogenates were prepared in ice cold Tris–Mannitol buffer (2 mM Tris, 50 mM Mannitol, pH 7.2) using mechanically driven Teflon fitted Potter–Elvejhem type homogeniser for few minutes till total disruption of cells. Homogenates were centrifuged at 10,000 \( \times g \) for 10 min at 4 \(^\circ\)C. Pellets were discarded and supernatant was used for various biochemical estimations.

2.8. Antioxidant defense system enzymes and lipid peroxidation

Total lipid peroxidation levels, and several key enzymes that indicate the antioxidant status of animals in different stress conditions were analyzed using established biochemical procedures as follows: lipid peroxidation (Wills, 1966), reduced glutathione (GSH) (Ellman, 1959), catalase (Luck, 1954), superoxide dismutase (SOD) (Kono, 1978), glutathione reductase (GR) (Carlberg and Mannervik, 1985), glutathione-S-transferase (GST) (Habig et al., 1974) and protein (Lowry et al., 1951).

2.9. Estimation of colonic zinc levels

The elemental analysis of zinc was carried out using Energy Dispersive X-Ray Fluorescence technique (EDXRF) at UGC-DAE CSR, Kolkata, India. EDXRF technique is most suitable analytical method to analyze trace elements, as it is a non-destructive method, has a high degree of sensitivity (in ppm) and allows multi-elemental analysis from each sample.

2.9.1. Sample preparation for EDXRF

The colonic tissues from all the animals were oven dried at 70 \(^\circ\)C to a constant weight and then ground with the help of agate pestle and mortar. Dried tissue powders (100 mg) so obtained were weighed and self-supporting pellets were made using a specially designed pure steel dye and a hydraulic press. A force of approximately 45 kN (kilo Newtons) was applied to the dye in order to make uniform thickness pellets.

2.9.1.1. EDXRF setup. In the present study, the tissue pellets were analyzed using Jordan Valley EX-3600 EDXRF spectrometer for the determination of zinc levels. The EX-3600 spectrometer involved a 50 W anode X-ray tube as an excitation source. The power of the X-ray tube was adjusted on-line for each individual measurement by the spectrometer software to secure optimum acquisition parameters for the current analysis. A Si(Li) detector coupled with computer was used to collect the fluorescent X-ray spectra from each sample. The X-ray tube, secondary exciter, target and the Si(Li) detector were placed in a tri-axial geometry mode. This geometry was used to minimize the background due to scattered photons. The EX-3600 spectrometer was pre-calibrated with standards that permitted evaluation of elemental concentrations present in unknown samples within an accuracy of ±5%.

2.10. Histopathological studies

For the histopathological observations at light microscopic level, fresh tissue pieces of colon were immersion fixed in buffered formalin. Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax. Blocks were made and 5–7 \( \mu \)m thick sections were double stained with hematoxylin and eosin and observed under light microscope.

2.11. Statistical analysis

The statistical significance of the data has been determined using one-way analysis of variance (ANOVA) and a multiple

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats examined</th>
<th>Colon tumor incidence (percentage of tumor bearing rats)</th>
<th>Colon tumor multiplicity (mean tumor/animal)</th>
<th>Tumor size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0.988 ± 0.185</td>
</tr>
<tr>
<td>DMH</td>
<td>6</td>
<td>100</td>
<td>2.5</td>
<td>0.680 ± 0.130y</td>
</tr>
<tr>
<td>Zinc</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0.988 ± 0.185</td>
</tr>
<tr>
<td>DMH + zinc</td>
<td>6</td>
<td>66.6</td>
<td>1</td>
<td>0.680 ± 0.130</td>
</tr>
</tbody>
</table>

Data of tumor size are expressed as mean ± S.D.

\( y \) P < 0.01 by Newman–Keuls test when values of Group IV are compared with Group II.
Table 2
Effect of zinc on body weight and ACF number of animals subjected to DMH treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight (g)</th>
<th>Number of ACF/colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>252 ± 23</td>
<td>–</td>
</tr>
<tr>
<td>Dimethylhydrazine (DMH)</td>
<td>212 ± 16b</td>
<td>19.3 ± 4.6</td>
</tr>
<tr>
<td>Zinc</td>
<td>272 ± 33</td>
<td>–</td>
</tr>
<tr>
<td>DMH + zinc</td>
<td>256 ± 10y</td>
<td>9.7 ± 3.3y</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± S.D. bP < 0.05 and yP < 0.01 by Newman–Keuls test when values of Group IV are compared with Group II.

3. Results

The results obtained from various experiments conducted in this study are depicted in Tables 1–4. The data from various treatment groups have been compared with the normal control animals. However, results obtained from zinc + DMH treated group were additionally compared with that of DMH group (Group II) as well.

3.1. Mortality

During this study, we did not observe any evidence for mortality as all the animals survived during the entire course of the experiment.

3.2. Colon tumor analysis

In the carcinogen Group II, tumor incidence was 100%. Administration of zinc at a dose level of 227 mg/L drinking water to DMH treated animals resulted in reduction in tumor incidence (Table 1) 66.6% when compared to DMH control Group II. The average number of tumors per tumor bearing rat (tumor multiplicity) was also considered in the study. Additionally, rats treated with DMH + zinc in Group IV showed a decline in tumor multiplicity compared to rats administered with DMH only (Table 1). In addition, a significant reduction in colon tumor size was also evident in Group IV rats when compared to those in carcinogen control Group II. There were no changes observed in Group III animals, given zinc alone treatment.

3.3. ACF number

ACF were stereoscopically distinguished from normal crypts by their darker staining and larger size,
Table 4
Zinc levels in the colon of animals subjected to different treatment (16 weeks)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Zinc (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>112.32 ± 18.26</td>
</tr>
<tr>
<td>Dimethylhydrazine (DMH)</td>
<td>80.96 ± 7.62a</td>
</tr>
<tr>
<td>Zinc</td>
<td>178.93 ± 25.27c,z</td>
</tr>
<tr>
<td>DMH + zinc</td>
<td>106.44 ± 18.04x</td>
</tr>
</tbody>
</table>

*aP < 0.05, *P < 0.01 and *cP < 0.001 by Newman–Keuls test when values are compared with control group. *dP < 0.05, *P < 0.01 and *zP < 0.001 by Newman–Keuls test when values of Group IV are compared with Group II.

3.4. Body weight changes

The variations in the body weights of the animals subjected to different treatments are shown in Table 2. The body weights of the normal control and zinc treated rats increased progressively throughout the study. However, DMH treatment resulted in a significant decrease in the body weights (P < 0.01), when compared to the normal control rats. Zinc treatment to DMH treated rats tended to improve the body weight growth in comparison to DMH treated animals (P < 0.05). Additionally, the daily food and water intakes were measured, and it was found that on an average 20–30 mL of water was consumed by each animal/day. However, no significant changes in food consumption were observed among various groups of animals.

3.5. Antioxidant defense system enzymes and lipid peroxidation

A statistically significant decrease (P < 0.001) in the levels of MDA was observed in the colons of rats subjected to 16 weeks of DMH treatment (Table 3). Simultaneous zinc treatment to DMH treated rats resulted in a significant increase in MDA levels. Similarly, zinc treatment alone to Group IV animals significantly reversed (P < 0.01) the otherwise increased lipid peroxidation levels when DMH treated animals were compared with zinc + DMH group at the end of the study.

DMH treatment to normal control animals resulted in a significant increase (P < 0.01) in the levels of GSH and GR enzyme activity (Table 3). On the contrary, a significant decrease in the activities of GST, SOD and catalase was observed following DMH treatment (P < 0.001). However, zinc treatment to DMH treated animals resulted in significant elevation in the levels of GSH and GR, as well as a simultaneous attenuation of activities of GST, catalase and SOD enzymes when compared with DMH treated group.

3.6. Zinc levels

The bio-availability and steady-state levels of zinc in colonic tissues following 16 weeks of DMH treatment were investigated. We observed a significant decrease (P < 0.05) in tissue concentrations of zinc in the colon following DMH treatment, when compared to the animals in the control group (Table 4). Animals in zinc + DMH treated group responded by recovering the zinc levels, in comparison to the DMH group. Further, a significant increase in zinc levels were observed in the colon of the animals treated with zinc alone (P < 0.001) in comparison to the normal animals.

3.7. Histopathology

Tissue sections of Group I animals displayed normal colonic architecture with no signs of apparent abnormality (Fig. 1). In the carcinogen treated Group II, well differentiated signs of dysplasia were evident. Nuclei were enlarged, thickening of epithelium was seen, cells were hyper-chromatic and showed increased mitotic activity. Simultaneously, there was a loss in nuclear polarity (Fig. 2). The tumor section of the colon from DMH treated rats also revealed the signs of poorly differentiated adenocarcinoma (Fig. 3). In the combined treatment group (Group IV), histoarchitecture revealed
Fig. 2. Showing the altered colonic histoarchitecture from DMH treated rats.

Fig. 3. Showing the altered colonic histoarchitecture from tumor section of DMH treated rats.

Fig. 4. Showing the colonic histoarchitecture from zinc treated rats.

Fig. 5. Showing the colonic histoarchitecture from DMH + zinc treated rats.

Fig. 6. Showing the colonic histoarchitecture from tumor section of DMH + zinc treated rats.

no signs of dysplasia but indicated a little loss of nuclear polarity (Fig. 4). The size and shape of the cells were uniform and the cells regained the near normal histoarchitecture (Fig. 5). Occasionally, hyper-chromatic nucleus were evident. However, well-differentiated ade-

nocarcinoma was evident in the tumor section of the colon from combined treatment group (Fig. 6). There were no signs of dysplasia or toxicity observed in Group III rats administered with zinc supplementation and displayed normal colonic histoarchitecture (Fig. 3).

4. Discussion

The inhibitory effects of zinc on the histological changes and oxidative stress enzymes were observed in an experimental model of DMH-induced colon carcinogenesis. The study clearly indicates that the administration of zinc in the presence of the pro-carcinogen DMH appreciably attenuates the alterations in the tissue levels of lipid peroxidation and the overall antioxidant enzymatic status. Further, the histological findings clearly support these biochemical data and suggest that zinc may play a promising anticancer role with respect to colon carcinogenesis.
Treatment of rats with zinc for 16 weeks caused a decrement in the tumor incidence, tumor multiplicity, with a concomitant reduction in average tumor size, strongly suggesting the potentiality of zinc in inhibiting/slowing tumorgenesis in the rat colon. Moreover, no tumor incidence in zinc alone treated group suggests that zinc at this dose level, causes no disruption of normal cellular homeostasis and hence it is non-toxic.

It is recognized that colon carcinogenesis is a multistep process that includes sequential selection and propagation of preneoplastic lesions. Several studies investigating the genotypic, morphological and growth features of ACF have supported the contention that ACF are preneoplastic lesions (Bird, 1995). The ACF system is frequently used to identify and study the modulation of colon carcinogenesis. The results of the present study indicated that zinc significantly inhibited the formation of ACF suggesting the potential of zinc in suppressing the 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 zinc 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 (Kawamori et al., 1995).

The decreased lipid peroxidation in the colon following DMH treatment is based on the assessment of the levels of reactive molecule MDA formed during the lipid peroxidation chain reaction. Previous studies have shown reduced rates of lipid peroxidation in the tumor tissue of various types of cancers (Cheeseman et al., 1986; Tanaka, 1997; Tanaka et al., 1998; Pillai et al., 1999). On the contrary, it has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes (Seven et al., 1999). There are contradictory results on this subject in the literature with regard to cancerous conditions. Huang et al. (1999) reported significantly increased lipid peroxidation, measured as MDA, in the serum of breast cancer patients. Seven et al. (1999) and Samir and El Kholy (1999), in their studies on patients with laryngeal carcinoma also reported that MDA levels were significantly increased compared with healthy controls. On the other hand, Gerber et al. (1997) reported that MDA levels decreases with increasing tumor size and progression in breast cancer. Our results provides a support to earlier findings of decrease in the levels of MDA, that could be a result of increased cell proliferation, which is thought to be involved in the pathogenesis of colon cancer. Cancer cells acquire particular characteristics that benefit their proliferation (Schmelz et al., 2000), and they tend to proliferate faster when the lipid peroxidation levels are low. Therefore, the decreased colonic lipid peroxidation observed in DMH treated rats could be due to increased proliferation. Thus, malignant tissues are less susceptible and more resistant to free radical attack, and hence lipid peroxidation is less intense (Nakagami et al., 1999). In addition, the decreased levels of lipid peroxidation in DMH treated rats may also be due to increased resistance and/or decreased susceptibility of the target organs to free radical attack. Interestingly, simultaneous zinc treatment to DMH treated animals showed an increase in the levels of MDA. The observed attenuated levels of LPO by zinc could be owed to the fact that it has a role in countering the cell proliferation, thereby, indirectly regulating the levels of LPO. However, the exact mechanism could not be ascertained from the present study and needs further exploration with regard to regulation of LPO by zinc in colon carcinogenesis.

Decreased lipid peroxidation associated with enhanced GSH in the colon and intestines is a well-known phenomenon in experimental carcinogenesis (Pillai et al., 1999). In our study we have also observed enhanced GSH levels following 16 weeks of DMH treatment. This may be due to the increased cell proliferation involved in the pathogenesis of DMH-induced colon cancer (Cao et al., 1997). It was previously demonstrated that GSH is expressed in greater amounts in the neoplastic cells, conferring a selective growth advantage (Obrador et al., 1997). It has also been reported that DMH treatment results in an increased tissue GSH content (Nijhoff and Peters, 1992). In the presence of GSH as a substrate and GPxs and GST as detoxifying enzymes, conjugation of toxic electrophiles with GSH takes place, conferring a selective growth advantage to cancer cells. Thus, the elevated GSH levels in colon as observed in our study can be used as a marker of cell proliferation. Interestingly, treatment with zinc to DMH treated animals modulated the levels of GSH, thus ascribing their protective effect in restoring GSH activity. Further, the results of increased GSH levels are in accordance with the findings of increased levels of glutathione reductase and decreased levels of glutathione-S-transferase.

The antioxidant enzymes SOD and catalase limit the effects of oxidant molecules on tissues and are activated in the defense against oxidative cell injury by means of their being free radical scavengers (Kyle et al., 1987). These enzymes work together to eliminate active oxygen species and small deviations in physiological concentrations may have a dramatic effect on the resistance
of cellular lipids, proteins and DNA to oxidative damage (Mates and Sanchez-Jimenez, 1999). In the present study, SOD and catalase activities were found to be significantly decreased following 16 weeks DMH treatment, when compared to the normal control animals. The decreased activities of SOD and catalase are due to a natural cellular response against the decreased levels of reactive oxygen species with regard to hydrogen peroxide and superoxide radicals.

The histopathological observations amply imply that supplementation of zinc under the experimental conditions can greatly affect the post-initiation stages of colon carcinogenesis by altering the efficacy at which DMH can initiate histological changes. Well-differentiated signs of dysplasia were observed in colonic tissue sections by DMH administration alone. Treatment with zinc greatly restored normalcy in the colonic epithelial cells, with no apparent signs of dysplasia. The ability of zinc to restore the histological changes induced by DMH indicates the anti-carcinogenic potential of this trace metal.

The present study observed a significant depression of colon zinc levels following DMH treatment, which is in line with some of the previous studies (Reddy et al., 2004). Similar observations were recently reported for lower zinc levels in prostate cancers as well (Franklin et al., 2005). Additionally, lowered zinc levels in the leukocytes in a variety of neoplastic diseases have been proposed to be a potential prognostic marker for cancer detection (Szmigielski and Litwin, 1964). Although the precise mechanistic role of zinc in human carcinogenesis is elusive, our current observations together with the published literature for the diminished zinc levels in colon cancer suggest that zinc directly or indirectly inhibits the growth of cancerous cells.

In conclusion, the results of this study suggest that zinc has a positive beneficial effect against chemically induced colonic preneoplastic progression in rats induced by DMH, which provides an effective dietary chemopreventive approach to disease the management. However, study needs further exploration with regard to other definitive bioassays including protein expression and documentation of specific molecular markers to establish the exact mechanism for zinc-mediated cancer chemoprevention.

References


