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BRIEF ARTICLE

Selenium as a chemopreventive agent in experimentally induced colon carcinogenesis

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Abstract

AIM: To elucidate the chemopreventive efficacy of selenium during experimentally induced colon carcinogenesis.

METHODS: Thirty-two male wistar rats were divided into four groups: group Ⅰ (normal control); group Ⅱ [1,2-dimethylhydrazine (DMH) treated]; group Ⅲ (selenium treated); and group Ⅳ (DMH + selenium treated). Groups Ⅱ and Ⅳ were given subcutaneous injections of DMH (30 mg/kg body weight) every week for 20 wk. Selenium, in the form of sodium selenite, was given to groups Ⅲ and Ⅳ at 1 ppm in drinking water ad libitum for 20 wk. At the end of the study, rats were sacrificed and their colons were analyzed for the development of tumors, antioxidant enzyme levels and histological changes.

RESULTS: 100% of the DMH treated rats developed tumors, which was reduced to 60% upon simultaneous selenium supplementation. Similarly, tumor multiplicity decreased to 1.1 following selenium supplementation to DMH treated rats. Levels of lipid peroxidation, glutathione-S-transferase, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) decreased following DMH treatment, whereas levels of glutathione (GSH) and glutathione reductase (GR) significantly increased in DMH treated rats. Selenium administration to DMH treated rats led to an increase in the levels of lipid peroxidation, SOD, catalase, glutathione-S-transferase and GPx, but decreased the levels of GSH and GR. Histopathological studies on DMH treated rats revealed dysplasia of the colonic histoarchitecture, which showed signs of improvement following selenium treatment.

CONCLUSION: The study suggests the antioxidative potential of selenium is a major factor in providing protection from development of experimentally induced colon carcinogenesis.

Key words: Colon cancer; Selenium; Antioxidant enzyme; Histopathology; Dimethylhydrazine

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INTRODUCTION

Colorectal cancer is amongst the leading causes of cancer-related deaths and one of the most commonly diagnosed cancers[1]. The oxidation of biomolecules due to reactive oxygen species (ROS) is associated with cellular dysfunction and leads to various biological responses, such as inflammation and apoptosis. When ROS attack DNA, oxidized bases are generated and the unrepaired oxidative DNA damage can induce mutations. Formation of hydroxylated bases of DNA is considered as an important event in chemical carcinogenesis[2,3]. Colon carcinogenesis is a multistep process in which oxygen radicals were found to enhance carcinogenesis at all stages: initiation, promotion, and progression[4]. The colon carcinogen 1,2-dimethylhydrazine (DMH) has been widely used to chemically induce colon cancers[5].

Dietary constituents have been reported to play vital roles in the development or prevention of cancer. Selenium, an essential trace nutrient, has been reported to improve immune function in animals[6,7], enhance neuropsychological function in humans[8] and ameliorate specific disease conditions in humans and animals[9]. The relationship between selenium and the etiology of cancer in humans remains elusive and intriguing, despite the number of studies published on the topic.

Selenium deficiency has been associated with initiation of events leading to the development of tumors[10,11]. Low levels of selenium have been associated with a higher risk of cardiovascular diseases and cancer in humans, which is another important factor related to dietary intake[12]. Epidemiological studies illustrate an increased incidence of colorectal cancer in humans in geographic regions where selenium is deficient[13]. Selenium affects colon cancer susceptibility and DNA methylation. Animals fed selenium-deficient diets had significantly hypomethylated colonic DNA compared with those fed diets supplemented with selenite or selenomethionine[14]. Thus, alterations in DNA methylation might help explain the increased tumorigenesis associated with selenium deficiency.

The present study was carried out to further explore the chemopreventive efficacy of selenium, if any, on the initiation and progression of colon cancer induced with DMH in a rat model.

MATERIALS AND METHODS

Chemicals

DMH, reduced nicotinamid adenine dinucleotide, glutathione (GSH), nitroblue tetrazolium, 5,5'-dithiobis 2-nitrobenzoic acid, were procured from Sigma-Aldrich (Delhi, India). Sodium selenite was purchased from E. Merck. All the chemicals used were of analytical grade.

Animals

Male wistar rats, in the weight range 120-150 g, were procured from the central animal house, Panjab University, Chandigarh. All the animals were housed in polypropylene cages under hygienic conditions. Basal supplemented diets (Ashirwad Industries, Punjab, India) were given to the animals. Before initiating the experiments, the animals were adapted to the laboratory conditions for a week. All the procedures were performed in accordance with the standard guidelines for care and use of laboratory animals and the protocols followed were approved by the Institute's Ethical Committee on animals.

Experimental design

Thirty-two animals were randomly and equally assigned into four treatment groups. Animals in Group I served as normal controls and were given water and diet *ad libitum*. Rats in this group were also administered with 1 mmol/L EDTA-saline subcutaneously per week, which was used as a vehicle for the DMH treatments. Animals in Group Ⅱ were given subcutaneous injections of DMH [dissolved in 1 mmol/L EDTA-normal saline (pH 6.5)] every week at 30 mg/kg body weight for 20 wk[15]. Group Ⅲ animals were given selenium in the form of sodium selenite in drinking water *ad libitum* at 1 ppm in drinking water. Animals in Group Ⅳ were given a combined treatment of DMH as well as selenium, similarly to Group Ⅱ and Group Ⅲ animals, respectively.

Record of body weights

A record of the body weights of normal control, DMH and selenium treated animals was kept throughout the study. The animals were weighed at the beginning of the experiment, once a week during the experiment and finally before sacrifice.

Colon tumor analysis

After 20 wk of DMH treatment, colons were excised from the rats, blotted dry, opened longitudinally and the inner surface was examined for visible macroscopic lesions. Tumors were easily discernable in the inflamed sections of the colon. The colons were observed for tumor incidence and multiplicity studies. Tumor size was recorded using a vernier caliper with 0.1 mm graduations. The chemopreventive tumor response was assessed on the basis of tumor incidence and multiplicity, which were calculated as follows: Tumor incidence, percentage of animals having tumors; Tumor multiplicity, mean of tumors counted/animals.

Preparation of colon homogenates

Animals from all the groups were sacrificed by cervical dislocation under light ether anesthesia at the end of the study. Their colons were removed and washed with ice chilled saline. Colon homogenates (10%) were prepared in ice cold Tris-Mannitol buffer (2 mmol/L Tris, 50 mmol/L Mannitol, pH 7.2) using a mechanically driven Teflon fitted Potter-Elvehjem type homogenizer for a few minutes to achieve total disruption of cells. Homogenates were centrifuged at 10 000 *g* for 10 min at 4℃. Aliquots of the supernatants were prepared and stored at -20℃ for various biochemical investigations.

Table 1 Effect of selenium on body weights of animals subjected to 20 wk of DMH treatment

Groups	Body weight (g)	
	0 d	20 wk
Ⅰ normal control	135 ± 15.00	282 ± 16.43
Ⅱ DMH	134 ± 16.73	210 ± 15.81[d]
Ⅲ selenium	134 ± 15.11	284 ± 37.81
Ⅳ DMH + selenium	134 ± 20.70	246 ± 33.61[a,b]

[b]$P < 0.01$ and [d]$P < 0.001$ by one-way ANOVA followed by LSD test when values are compared with normal control group; [a]$P < 0.05$ by one-way ANOVA followed by LSD test when values of group Ⅳ animals are compared with group Ⅱ animals. Values are expressed as mean ± SD.

Lipid peroxidation and antioxidant defense system enzymes

Lipid peroxidation was assayed according to the method of Wills[16]. One of the end products of lipid peroxidation is malondialdehyde (MDA), which forms a pink colored complex with thiobarbituric acid with an absorption maxima at 532 nm. Glutathione-Peroxidase enzyme activity was assayed using glutathione reductase and H_2O_2 as substrates, and the optical density was read at 340 nm with a double beam spectrophotometer[17]. The activity of total SOD was measured at 560 nm following the method of Kono[18]. The enzymatic determination of catalase was performed according to the method of Luck[19] and the concentration of H_2O_2 was monitored at 240 nm. The activity of glutathione-S-transferase was estimated according to the method of Habig *et al*[20]. Reduced GSH contents were determined using the method of Ellman[21]. Glutathione reductase (GR) activity was assayed using the method of Carlberg *et al*[22].

Histopathological studies

Formalin fixed tissues were processed for histopathological observations at the light microscopic level. Briefly, following an overnight fixation in buffered formalin, tissues were dehydrated through ascending grades of alcohol, cleared in benzene and embedded in paraffin. Sections of 5-7 micrometer thick were cut, placed serially on clean glass slides and then de-paraffinized through descending grades of alcohol. Sections were made from each colon tissue and were stained with hematoxylin and eosin. These were then observed under a light microscope and the gross morphology was noted.

Statistical analysis

The statistical significance of the data was determined using one-way analysis of variance (ANOVA) and a multiple post hoc test (LSD). The significance was set at $P < 0.05$. The results are represented as mean ± SD.

RESULTS

Body weight changes

The variations in the body weights of the animals subjected to different treatments are shown in Table 1. The body weights of all the normal and treated animals (Table 1) rose steadily throughout the study. However, the body weight gains of the animals treated with DMH was markedly less as compared to the normal controls. Selenium treatment of DMH treated rats tended to improve the body weight growth in comparison to DMH treated animals.

Table 2 Chemopreventive efficacy of selenium on the tumor incidence, tumor multiplicity and tumor size of DMH-induced rat colonic tumors

Groups	Colon tumor incidence (percentage of tumor bearing rats)	Colon tumor multiplicity (mean tumor/animal)	Tumor size (cm)
Normal control	0	0	-
DMH	100%	2.6	0.911 ± 0.196
Selenium	0	0	-
DMH + selenium	60%	1.1	0.609 ± 0.250[b]

[b]$P < 0.001$ by one way-ANOVA followed by LSD test when values of Ⅳ animals were compared with Group Ⅱ animals. Values are expressed as mean + SD.

Colon tumor analyses

Tumor incidence was observed to be 100% in DMH group of rats. Further, the tumor incidence was reduced in the DMH treated rats that were supplemented with selenium (Table 2). Similarly, tumor multiplicity that increased following DMH treatment, tended to decrease upon selenium supplementation. In addition, a significant reduction in colon tumor size was also evident in Group Ⅳ rats when compared to DMH treated rats.

Antioxidant defense system enzymes and lipid peroxidation

In the present study, MDA levels as a direct indicator of lipid peroxidation, were decreased significantly ($P < 0.001$) after 20 wk of DMH treatment. Selenium treatment to normal rats did not indicate any significant change in MDA levels. Selenium supplementation to DMH treated rats significantly reversed ($P < 0.01$) the otherwise altered levels of LPO observed at the end of the study.

DMH treatment to normal animals resulted in a significant decrease in the enzymes activities of GST, SOD, catalase and GPx ($P < 0.001$). In contrast, a significant increase ($P < 0.01$) in the levels of GSH and the enzyme activity of GR was observed following DMH treatment (Table 3). However, selenium treatment to DMH treated animals resulted in a significant elevation in the activities of enzymes GST, catalase, GPx and SOD, but caused a significant decrease in the levels of GSH and GR when compared with DMH treated group.

Histopathology

Histopathological analysis showed that the colon of rats from normal and selenium treated groups had normal histoarchitecture with no signs of apparent abnormality (Figure 1A and C). In the DMH treated groups, well differentiated signs of dysplasia were observed. Nuclei

Table 3 Effect of selenium on lipid peroxidation and antioxidant enzymes in the colons of rats subjected to 20 wk of DMH treatment

Groups	Lipid peroxidation (nmoles of MDA/ min/100 mg protein)	GR (mmol NADPH oxidized/ min/mg protein)	GST (μmol of conjugate formed/ min/mg protein)	SOD (I.U)	CAT (mmol of H_2O_2 decom posed/ min/mg protein)	GSH (μmol GSH/g tissue)	GPx (mmol NADPH oxidized/ min/mg protein)
Normal control	3.229 ± 0.38	1.375 ± 0.066	0.158 ± 0.073	6.430 ± 0.617	1.234 ± 0.200	0.629 ± 0.011	0.706 ± 0.02
DMH	2.237 ± 0.35[d]	1.708 ± 0.101[d]	0.033 ± 0.021[d]	4.438 ± 0.316[d]	0.794 ± 0.043[d]	1.089 ± 0.023[d]	0.434 ± 0.15[d]
Selenium	3.239 ± 0.20	1.334 ± 0.217	0.137 ± 0.027	6.287 ± 0.524	1.230 ± 0.113	0.637 ± 0.017	0.711 ± 0.015
DMH + selenium	3.032 ± 0.21[f]	1.546 ± 0.043[a,c]	0.069 ± 0.022[b]	5.695 ± 0.417[a,f]	1.029 ± 0.278[c]	0.646 ± 0.027[f]	0.614 ± 0.17[d,f]

[a]$P < 0.05$, [b]$P < 0.01$ and [d]$P < 0.001$ by one way-ANOVA followed by LSD test when values are compared with control group; [c]$P < 0.05$ and [f]$P < 0.001$ by one way-ANOVA followed by LSD test when values of Group Ⅳ animals are compared with Group Ⅱ animals. Values are expressed as mean + SD.

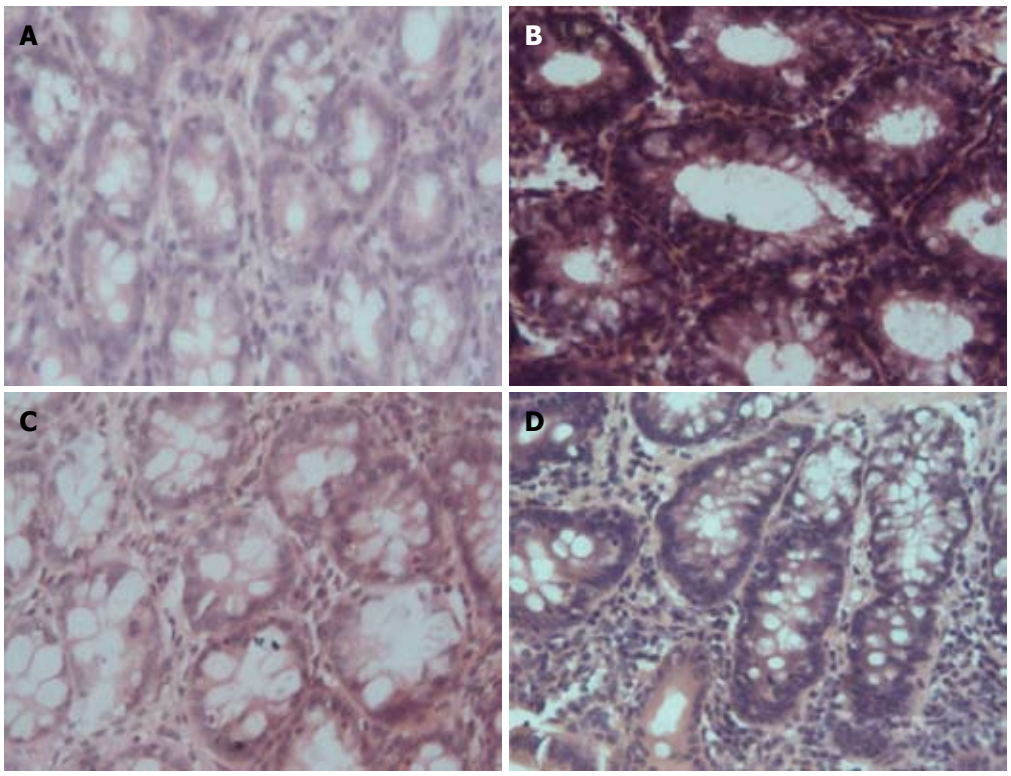

Figure 1 Histo-micrograph of rat colon. A: Normal histoarchitecture of rat colon (× 40); B: Altered colonic histoarchitecture from DMH treated rats (× 40); C: Colonic histoarchitecture from selenium treated rats (× 40); D: Colonic histoarchitecture from DMH + selenium treated rats (× 40).

were enlarged, thickening of epithelium was seen, cells were hyper-chromatic and showed increased mitotic activity. Simultaneously, there was a loss in nuclear polarity (Figure 1B). In the combined treatment group (Group Ⅳ), the histoarchitecture revealed no signs of dysplasia, but did indicate some loss of nuclear polarity (Figure 1D).

DISCUSSION

The protective effects of selenium on the histoarchitecture and oxidative stress enzymes were observed in an experimental model of DMH-induced colon carcinogenesis. The study clearly indicates that the administration of selenium attenuates the DMH induced alterations in the levels of lipid peroxidation and the overall antioxidant enzymatic status in the rat colons. Furthermore, the histological findings clearly support these biochemical data and suggest that selenium might play a promising anticancer role with respect to colon carcinogenesis. 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[23].

In the present study, selenium treatment to DMH treated rats for 20 wk caused a reduction in tumor incidence and tumor multiplicity, with a concomitant reduction in average tumor size. These data strongly suggest that selenium has the potential to inhibit/slow tumorigenesis in the rat colon. Moreover, the absence of tumor incidence in rats treated with selenium alone suggests that selenium, at this dose level, causes no disruption of normal cellular homeostasis and hence is non-toxic.

The levels of lipid peroxidation in the colon tissues were decreased after 20 wk of DMH treatment. Previous studies have shown reduced rates of lipid peroxidation in the tumor tissue of various types of cancers[24-27]. 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[28]. There are contradictory results on this subject in the literature with regard to cancerous conditions. Several investigators also reported that MDA levels were significantly increased in cancerous tissue when compared to healthy controls[29,30]. Devasena *et al*[31] reported increased, tumor incidence as well as enhanced LPO, in the circulation of colon tumor bearing rats. On the other hand, Gerber *et al*[32] reported that MDA levels decreased with increasing tumor size and progression in breast cancer. Certain studies have reported an inverse relationship between lipid peroxidation and cell proliferation[33]. Our results indicate that a decrease in the levels of MDA can be attributed to increased cell proliferation, which is thought to be involved in the pathogenesis of colon cancer. Cancer cells acquire particular characteristics that promote their proliferation[34] and tend to proliferate faster when the lipid peroxidation level is low. Therefore, the decreased lipid peroxidation observed in DMH-treated rats could be due to increased cell proliferation. The malignant tissues seem to be less susceptible and more resistant to free radical attack, and hence lipid peroxidation is less intense[35]. Interestingly, simultaneous selenium treatment to DMH treated animals showed an increase in the levels of MDA.The observed increased levels of LPO under selenium treatment could be as a consequence of the inhibitory action of selenium on the proliferative activity of cancerous cells. We also observed reduced catalase levels in DMH treated rats. The fall in catalase activity correlated well with tumor stage according to Dukes, suggesting that this peroxisomal enzyme could be used as a potential prognostic marker[36]. Decreased lipid peroxidation associated with enhanced GSH in the colon and intestines is a well known phenomenon in experimental carcinogenesis[37]. We have also observed enhanced GSH levels following 20 wk of DMH treatment. This might be due to the increased cell proliferation involved in the pathogenesis of DMH-induced colon cancer[38]. It was previously demonstrated that GSH is expressed in greater amounts in the neoplastic cells, conferring a selective growth advantage[39]. It has also been reported that DMH treatment results in increased tissue GSH content[40]. In the presence of GSH as a substrate and GPx 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 the colon, as observed in our study might be used as a marker of cell proliferation. Interestingly, treatment with selenium to DMH treated animals modulated the levels of GSH, thus ascribing their protective effect in restoring GSH activity. Furthermore, 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, GPx 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[41]. 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[42]. In the present study, SOD, GPx and catalase activities were found to be significantly decreased following 20 wk DMH treatment, when compared to the normal control animals. The decreased enzyme activities of SOD, GPx and catalase could be due to post-translational or oxidative modification of ROS scavenging enzymes[43]. The protective effect observed upon supplementation of selenium indicates that selenium eliminates the toxic effects of DMH on the activity of these enzymes.

Selenium is an essential component of several enzymes such as glutathione peroxidase (GSH-Px), thioredoxin reductase (TR) and selenoprotein P (SeP), which contains selenium as selenocysteine. Selenium is also essential for cell culture when a serum-free medium is used[44,45]. The antioxidant activity of selenium can be explained by its important role in preventing lipid peroxidation and in protection of integrity and functioning of tissues and cells. The role of selenium in preventing lipid peroxidation and oxidative damage has also been demonstrated in colon studies[44,46]. Moreover, Saito *et al*[45] have reported that selenium and Vit E show compensative effects and that a deficiency of both elements might cause massive injury. Selenium compounds are known for their antioxidative ability, therefore another favorable explanation is that selenium compounds affect carcinogen activation and metabolism through inhibition of phase I enzymes and induction of phase Ⅱ enzymes[47-49].This mechanism has been well documented to be important for the chemo-preventive activity of many thiol-reactive chemopreventive agents[50-53].

The ability of selenium compounds to inhibit growth and induce tumor cell apoptosis has been suggested to be a potential mechanism for cancer chemoprevention[47]. Amagase *et al*[54] and Ip *et al*[55] reported that selenium, supplied either as a component of the diet or as a constituent of a garlic supplement, enhanced protection against 7,12-di methylbenz[*a*]anthracene induced mammary carcinogenesis over that provided by garlic alone. Suppression in carcinogen bioactivation, as indicated by a reduction in DNA adducts, might account in part for this combined benefit of garlic and selenium[56].

The histopathological observations suggest that supplementation of selenium 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 selenium greatly restored the normal histoarchitecture in the colonic epithelial cells, with no apparent signs of dysplasia. The ability of selenium to restore the histological changes induced by DMH indicates the anti-carcinogenic potential of this trace metal. Increased antioxidant defense upon selenium

treatment could lower the ROS-mediated damage at the initiation as well as during progression/promotion phase of tumorigenesis. The antioxidative activity of organoselenium compounds is believed to be based on the prominent role that selenium plays in many of the enzymes of the oxidative defense system[57-61]. Studies have demonstrated that selenium supplementation reduces the incidence of cancer, particularly prostate cancer[62,63]. Evidence from experimental studies suggests that apoptosis is a key event in cancer chemoprevention by selenium and reactive oxygen species play a role in induction of apoptosis by selenium compounds. Xiang *et al*[63] found that selenite induces cell death and apoptosis by production of superoxide in mitochondria and activation of the mitochondrial apoptotic pathway and MnSOD plays an important role in protection against pro-oxidant effects of superoxide from selenite during proliferative phase. The data suggest that superoxide production in mitochondria is, at least in part, a key event in selenium-induced apoptosis in prostate cancer cells. Ganther[48] reported that the metabolism of selenium compounds is a prerequisite for cancer prevention. Extensive studies have concluded that selenium compounds directly converted to mono-methylated forms, (methylselenol, CH3SeH) or related intermediates (e.g. aromatic selenol) are powerful chemopreventive agents. The possible mechanisms by which selenium is postulated to decrease the incidence of cancer include inhibition of oxidative damage to DNA, recharging of cellular proliferation, modulation of apoptosis, and alteration of cellular gene expression.

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

COMMENTS

Background

The process of carcinogenicity presents a major challenge to scientists. Cancer chemoprevention refers to the use of pharmacological agents to inhibit, delay or reverse the multi-step process of carcinogenesis. The last two decades in particular have witnessed explosive growth in this emerging field of cancer chemoprevention. Many classes of agents include antioxidants and other diets have shown promise as chemopreventive agents. Therefore, for reducing the incidence of cancer, modifications in dietary habits, especially by increasing consumption of fruits and vegetables rich in antioxidants are increasingly advocated. However, in the present study, selenium has been proposed as chemopreventive agent to reduce the incidence of cancer in an animal model. Biochemical and histological techniques used to detect the changes in the antioxidant activity and microscopic alterations in the colon tissues of the animals has been reported.

Research frontiers

The alteration of antioxidant enzymes activity can be analyzed by biochemical estimation and histopathological study when colon cancer takes place because of the toxicity of pro-carcinogen 1,2-dimethylhydrazine (DMH). The results of present article will be helpful for carrying out further studies concerning chemopreventive role of selenium.

Innovations and breakthroughs

In the present study, results from both biochemical assays and histological study showed reactive oxygen species increase in the rats due to pro-carcinogen DMH. Selenium supplementation to the DMH treated animals changed the altered levels of antioxidant enzymes due to its important antioxidative property.

Applications

This study is useful to explain the anti-oxidative/anti-tumor activity of selenium. It might also play an important role in the treatment of tumors.

Peer review

It is widely known that selenium can inhibit colon carcinogenesis induced by repeated injection of DMH, but the precise mechanisms remain to be investigated. The authors provided evidence to indicate that selenium can exert anti-oxidant activities and eventually protect this colon carcinogenesis. The observations are novel and merit publication.

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