RESEARCH ARTICLE

Possible Protective Effects of Quercetin and Sodium Gluconate Against Colon Cancer Induction by Dimethylhydrazine in Mice

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Abstract

Micronutrients in food have been found to have chemopreventive effects, supporting the conclusions from epidemiologie studies that consumption of fresh fruits and vegetables reduces cancer risk. The present study was carried out to evaluate the role of querctin (Q) and sodium gluconate (GNA) supplementation separately or in combination in ameliorating promotion of colon tumor development by dimethyl-hydrazine (DMH) in mice. Histopathological observation of colons in mice treated with DMH showed goblet cell dysplasia with inflammatory cell infiltration. This pathological finding was associated with significant alteration in oxidative stress markers in colon tissues and carcinoembryonic antigen (CEA) levels in plasma. Mice co-treated with GNA and Q showed mild changes of absorptive and goblet cells and inflammatory cell infiltration in lamina properia, with improvement in oxidative stress markers. In conclusion, findings of the present study indicate significant roles for reactive oxygen species (ROS) in pathogenesis of DMH-induced colon toxicity and initiation of colon cancer. Also, they suggest that Q, GNA or the combination of both have a positive beneficial effect against DMH induced colonic cancer induction in mice.

Keywords: Colon cancer model - oxidative stress - histopathology - Q - GNA

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Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (Mathers et al., 2008). Colorectal cancer (CRC) is the third most common cancer worldwide after lung and breast cancers (Gado et al., 2014). Epidemiologic studies reveal an relationship between diet and the prevalence of CRC, which can be promoted by a diet rich in fat and meat (Aggarwal, 2008).

1,2-dimethylhydrazine (DMH) has been widely used as a potent colon carcinogen in experimental models because of the similarity between this model and human diseases pathology (Wang et al., 2011). DMH is metabolized in liver to azoxymethane and methylazoxymethanol which is further transported to colon and elicits oxidative stress by methylating biomolecules of colonic epithelial cells leading to inflammation and tumor promotion (Hamiza et al., 2012). Exposure to reactive oxygen species (ROS) can induce DNA damage, leading to genetic lesions that initiate tumourigenicity and subsequent tumour progression (Venkatachalam et al., 2013). DMH enhances the lipid peroxidation rate and increases the activity of enzymes that metabolize toxin in liver and colon (Dadkhah and Fatemi, 2010).

Several studies focused on the importance of pro- and anti-oxidant indicators, as markers of chemoprevention for many cancers, by natural and synthetic compounds (Balasenthil et al., 2000; Devasena et al., 2006; Ghadi et al., 2009). One of these antioxidants is Quercetin (Q), which increased the sensitivity of resistant colorectal tumors with microsatellite instability to the chemotherapy drug, 5-fluorouracil (Xavier et al., 2011), suppressed azoxymethane-induced hyperproliferation and focal hyperplasia in the colon (Deschner et al., 1991), and reduced intestinal tumor formation (Akagi et al., 1995). However, other studies indicated that Q supplementation accelerates colon tumor formation (Pereira et al., 1996; Mahmoud et al., 2000). The possible explanations for these discrepancies among the results of studies that were conducted on Q include the prooxidant activity of the flavonoid's oxidized form (Sahu and Washington, 1991). In general, supplementing prebiotic enhances growth performance and improved feed utilization by promoting beneficial bacteria and inhibiting pathogenic bacteria (Fuller and Peridigón, 2008). Sodium gluconate (GNA) promoted growth performances of nursery pigs by increasing intestinal beneficial bacteria population and

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intestinal morphology (Davis et al., 2002; Poeikhampha et al., 2007; Fuller and Peridigón, 2008). Hence, gluconic acid can be used as a prebiotic, stimulating butyrate production in the large intestine (Tsukahara et al., 2002). Butyrate is the main energy source for epithelial cells of the large intestine (Scheppach et al., 1995), stimulates mucous release (Shimotoyodome et al., 2000), and mineral and water absorption from the intestinal lumen (Holtug et al., 1995). Moreover, the combination of butyrate and aspirin had an enhanced effect on apoptosis in human colorectal cancer cells (Menzel et al., 2002). Thus, our aim was to evaluate the protective effect of Q and GNA individually or the combination of both supplementations against DMH induced oxidative stress and promotion of colon cancer in male mice.

Materials and Methods

Chemicals

1,2-Di methylhydrazine, quercitin, sodium gluconate, N, N diphenyl-p-phenylenediamine, superoxide dismutase, epinephrine, thiobarbituric acid (TBA), naphthylethylene diamine dihydrochloride, 5,5 dithiobis (2-nitrobenzoic acid (DTNB), triton-X100, sulfanilamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA. All other chemicals and reagents were of the highest purity commercially available.

Animals

Sixty adult male albino mice weighing 25-30 grams were obtained from the Animal House of the Faculty of Medicine, Assuit University, Assuit, Egypt. Mice were housed in cages and were kept in a room temperature (30±2ºC) with normal 12 h light/12 h dark cycle in animal room, Zoology Department, Faculty of Science, Assiut University. They were allowed to acclimatize for one week before the experiments. Animals were divided into 5 groups of 12 mice each. Group I: served as a control group and the other 4 groups were injected intraperiteoneally (i.p.) with a dose of DMH (25 mg/Kg b.w.) once a week for 5 weeks according to (Hamiza et al., 2012). Then, GII was left as positive control, however, group III, IV and V were co-treated daily for 5 weeks with Q (50 mg/kg b.w.) according to Ragab et al. (2014), GNA (50 mg/kg b.w) according to Kameue et al. (Kameue et al., 2004), and the combination of Q (50 mg/kg b.w.) and GNA (50 mg/Kg b.w.), respectively. All animals received human care and the study protocols were in compliance with Institutional Guidelines for the use of laboratory animals.

Collection and preparation of samples

Mice of different groups were killed by cervical dislocation after anesthesia with ether, blood samples was collected for the hematological measurements and colon was quickly removed and small piece fixed in 10% neutral buffered formalin for histopathological study. The other part was first frozen with liquid nitrogen then stored at -20°C to be used for biochemical studies. 10% w/v homogenates in 0.1 M phosphate buffer (pH 7.4) were prepared using IKA Yellow line DI homogenizer (18 Disperser, Germany). The homogenates were centrifuged at 6000 rpm for 1 h at 4°C and the supernatant cytosols were kept frozen at -20°C for the subsequent biochemical assays.

Analytical measurements

Blood picture for each mouse was performed by haematological analizar (media serve, exigo haematology analizar). Quantutative determination of CEA concentration in plasma was performed by ELISA kit catalogue No.PT-CEA-96 (European authorized representative JTC Diagnosemittel UG Schulweg 8 D-34516 Voehl/Germany) based on the principle of a solid phase enzyme-linked immunosorbant assay. The total protein content in colon tissue was determined colormitically using the method of Lowry et al. (1951). Malondehydride (MDA) level in colon tissue was estimated by the method of Ohkawa et al., (1979) using thiobarbituric acid (TBA) which reacts with MDA to form a stable pink color. Colon content of glutathione (GSH) levels was measured according to Beutler et al. (1963) based on the reduction of DTNB with GSH to produce a yellow compound (reduced chromogen) that is directly proportional to GSH concentration. Super oxide dismutase (SOD) activity was assessed by the method of Misra and Fridovich (Misra and Fridovich, 1972) which is based on the inhibition of the autoxidation of epinephrine at alkaline medium. Catalase activity (CAT) was determined according to the procedure of Luck (1963), based on its ability to decompose hydrogen peroxide. The activity of glucose-6-phosphte dehydrogenase (G6PD) was determined according to the ability to reduce NADP according to the method of Haghighi et al. (1998).

Histopathological examination

Specimens of colon were taken from mice of different groups directly after scarification. They were fixed in 10% neutral buffer formalin, embedded in paraffin, sectioned at 5 μ and stained with hematoxylin and eosin stain (H&E). The sections were examined using the light microscope (Bancroft and Gamble, 2008). Stained sections were examined under light microscope (Olympus CX31, Japan) and photographed using a digital camera (Olympus, Camedia C-5060, Japan).

Statistical analysis

The results were analysed using one way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test and graph-pad prism program for windows, version 3.0 (Graph pad software, Inc, San Diago CA. USA). Levels of significance between the groups were accepted at $p<0.05$, 0.01 or 0.001, and the data were expressed as mean ± Standard error (SE).

Results

After 5 weeks of treatment with DMH, mice exhibited alterations in the hematological parameters as compared to controls as shown by decrease in erythrocytes count and increase in leukocytes count especially lymphocytes and esinophils. Co-treatment of mice with GNA, Q or the combination of GNA and Q modulate these alterations by elevating RBCs count significantly while only the

Table 1. Hematologic Parameters of Control and Different Treated Mice

DMH+GNA+Q	DMH+Q	DMH+GNA	DMH	Control		
4.222±0.10b*	5.060±0.42[b]**	4.283±0.31[b]*	3.168±0.22[a]**	5.527±0.78	RBCs	(106 mm-3)
9.743±0.66b*	7.000±0.63	8.967±0.65	6.380±0.67[a]***	11.54±1.03	WBCs	(103 mm-3)
1.886±0.22b**	1.900±0.42[b]*	1.200±0.15	0.767±0.04	1.017±0.13	Neutrophils	(103 mm-3)
10.05±0.43b***	4.667±0.23	3.933±0.25	3.950±0.49[a]*	9.250±0.88	Lymphocytes	(103 mm-3)
0.975±0.13b*	0.900±0.13	0.717±0.06	0.500±0.03	0.800±0.09	Monocytes	(103 mm-3)
0.471±0.04	0.383±0.04b*	0.450±0.05	0.583±0.04[a]**	0.217±0.03	Esinophils	(103 mm-3)

Values represent means ± SE. a: significant difference between control and DMH group. b: significant difference between DMH and different treatments. * P < 0.05, ** P < 0.01, *** P < 0.001

Table 2. Oxidative Stress Markers in Colon Tissue and Tumor marker CEA in Plasma of Control and Different Treated Mice

DMH+GNA+Q	DMH+Q	DMH+GNA	DMH	Control	
0.719±0.06b**	0.688±0.05b**	1.289±0.17	1.465±0.14a**	0.79±0.13	LPO (nmol/mg protein)
0.539±0.09b**	0.774±0.17b**	0.897±0.064b**	7.011±2.10a*	3.44±1.37	NO (nmol/mg protein)
16.41±0.88b***	37.19±5.40	18.38±1.18b***	96.47±16.63a*	135.7±33.52	CAT (U/mg protein)
0.561±0.04b***	1.418±0.14b***	0.823±0.04b***	7.497±0.63a***	4.03±0.80	SOD (U/mg protein)
4.58±0.43b***	14.27±3.07b***	7.68±1.31b***	71.72±10.07***	14.90±1.17	G6PD (mU/mg protein)
0.104±0.008b***	0.127±0.01b***	0.091±0.006b***	0.597±0.10b***	0.30±0.05	GSH (µg/ mg protein)
0.35±0.06b***	1.32±0.13b***	2.0±0.39b**	3.1±0.17a***	0.575±0.155	CEA (ngm/ml plasma)

Values represent means ± SE. a: significant difference between control and DMH group. b: significant difference between DMH and different treatments. *P<0.05, **P<0.01, ***P<0.001

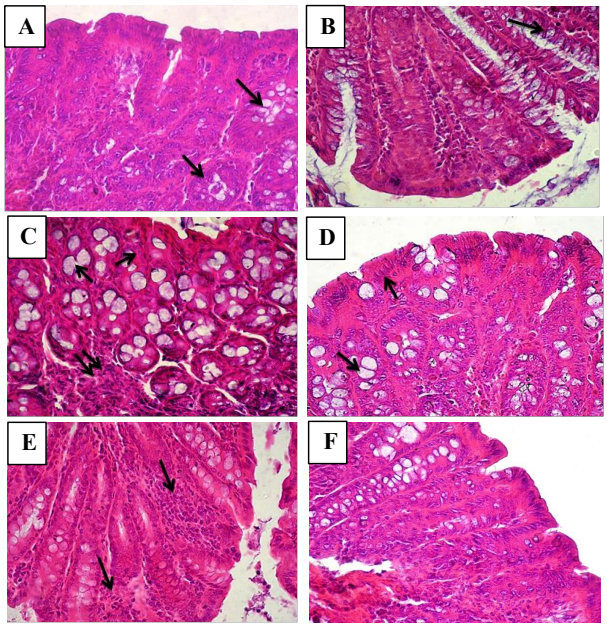

Figure 1. Representative Photomicrographs of Colon Tissue (H&E x400). A: Control group showing normal flat mucosa punctuated by numerous straight tubular crypts. B&C: DMH treated group showing goblet cells dysplasia (arrow) with inflammatory cells infiltration (double arrow). D: DMH + GNA treated group showing mild changes of absorptive and goblet cells (arrow). E: DMH + Q treated group showing inflammatory cells infiltration in lamina properia. F: DMH + GNA + Q treated group showing more or less changes as compared with other treated groups

combined treatment showed a significant increase of leukocytes count especially lymphocytes as shown in table (1).

Biochemical analysis of colon tissue cleared that DMH caused a significant increase in the levels of LPO, NO, and GSH and in the activities of SOD and G6PD, however, CAT activity was significantly decreased. Co-treatment of mice with GNA, Q or the combination of GNA and Q modulated these changes by reducing LPO, NO, CAT, SOD, GSH, G6PD and CEA significantly by different levels as compared with DMH treated groups (Table 2).

Histopathological study cleared that DMH exhibited dysplastic goblet cells with irregular shape and size with inflammatory cells infiltration (Figs. 1B, C) as compared with control rats (Figure 1A). Co-treatment with GNA showed mild changes of absorptive and goblet cells (Figure 1D). Quercetin treatment revealed inflammatory cells infiltration in lamina properia (Figure 1E). While, combined treatment with GNA and Q showed more or less changes as compared with other treated groups (Figure 1F).

Discussion

The role of the immune system in controlling tumor development has been reported by Chung et al. (2003). In the present study DMH induced an alteration in the haematological status of mice as seen by a significant decrease in the count of RBCs, WBCs, neutrophiles, lymphocytes, and monocytes. However, the eosinophils count was significantly increased in comparison with the control. It is known that oxidative stress in erythrocytes play a role in haematological abnormalities and progression of many disease like carcinogenesis (Childress, 2012). In this topic Jrah-Harzallah et al. (2013) found that DMH induced oxidative damage and reduction in erythrocytes count and hemoglobin level due to inhibition of RBC production and/or erythrocyte destruction in hemopoietic organs.

In the present study, the significant decrease in the leukocytes especially neutrophils and lymphocytes and platelets in DMH treated mice because leukocytes migrate to inflammation site and their attraction is facilitated by platelets (Laoui et al., 2011; Vieira-de-

Abreu et al., 2012). Moreover, neutrophils as the first line of defense are rapidly recruited to inflamed loci in response to inflammatory mediators released at the injury site (Malawista et al., 1992; Coussens and Werb, 2002). Also, lymphocyte depletion with consequent depression of innate cellular immunity is a severe clinical problem that can develop during cancer progression (Tavares-Murta and Murta, 2008). In the present study co-treatment of rats with Q, GNA or the combination of both restored the hematological impairments as compared with DMH-treated mice because antioxidant supplementation has been reported to increase natural killer cells which exhibit cytotoxic activity against tumor cells (Chung et al., 2003).

Carcinoembryonic antigen (CEA) is a tumour marker widely measured in colorectal cancer (Duffy et al., 2007). In the present study plasma CEA level was significantly increased in DMH treated mice, however, co-treatment of mice with Q, GNA or the combination of both normalized CEA level in plasma. It is known that CEA in colon cancer plays an important role in tumor invasion and metastasis duo to its functions as a promoter of cellular aggregation, regulator of the innate immune system, and mediator of signal transduction (Hammarström and Baranov, 2001; Li et al., 2010).

DMH is metabolized to a methyl free radical and generates hydroxyl radical or hydrogen peroxide in the company of metal ions that may contribute to initiation of cancer. In the present study, administration of DMH to mice caused a significant increase in lipid peroxidation in colon tissue. Lipid peroxidation is marker of oxidative damage and its elevation has been found after treatment with DMH (Sengottuvelan et al., 2006; Hamiza et al., 2012). Co-treatment of mice with Q, GNA or the combination of both significantly decreased lipid peroxidation level as compared to DMH-treated mice. Similarly, tanic acid and curcumin significantly attenuated elevated levels of lipid peoxidation in DMH induced colon cancer promotion (pereira et al., 1996; Hamiza et al. 2012).

Also, the present data showed that NO level was significantly increased in colon tissue of mice treated with DMH and decreased in colon of mice co-treated with Q, GNA or the combination of both in comparison with DMH treated mice. Similarly, El-Sayed et al. (2004) found an increase in plasma NO of DMH-induced colon cancer than control rat. This increase in NO level in plasma may be returned to the increase in inducible nitric oxide synthase as seen in colon tumor tissues of rats induced by azoxymethane (Rao et al., 1998). Moreover, the production of NO by endothelial nitric oxide synthase in endothelial cells of the neovasculature may cause vasodilation and increase blood flow to the tumor tissues to support their growth (Takahashi et al., 1997). In addition, supplementation or rats with Allium sativum significantly reduced the elevated levels of plasma NO in DMH-induced colon cancer (El-Sayed et al., 2004).

Elimination of free radicals in biological systems is achieved through enzymatic and non-enzymatic antioxidants, which act as major defense systems against free radicals (Nandhakumar et al., 2012). In the present study, GSH level was significantly increased in colon tissue of mice treated with DMH in comparison with the control and co-treatment of mice with Q, GNA and the combination of both results in more depletion of GSH content. However, Hamiza et al. (2012) and Ahmad and Sultana (Ahmad and Sultana, 2012) found a significant decrease in GSH levels in rats treated with DMH and treatment of rats with tannic acid restored the normal levels of GSH. Depletion of GSH level in colon tissue because DMH generates free radicals that scavenging by GSH and other enzymatic antioxidants like SOD and CAT (Sengottuvelan et al., 2006). Accordingly, in the present study, CAT activity was decreased; however, the activities of SOD and G6PDH were increased in colon tissue of mice treated with DMH. And co-treatment of rats with Q, GNA or the combination of both restored those activities to normal levels. In comparison, the activities of SOD and CAT were decreased in DMH treated rats and co-treatment with selenium led to an increase in the activities of SOD and CAT (Ghadi et al., 2009). Moreover, it is found that increased tumor incidence was accompanied by a significant decrease in the activities of SOD and CAT and administration of curcumin significantly decreased colon tumor incidence and enhanced SOD and CAT activities (Devasena et al., 2006).

The effects of DMH upon colonic proliferation were described as an increased proliferation rate, increased size of proliferation zone and deeper crypts (Chang et al., 1997; Ma et al., 2002). In the present study, DMH showed dysplastic goblet cells with irregular shape and size with inflammatory cells infiltration. Co-treatment of mice with GNA or combination of Q and GNA showed mild changes of absorptive and goblet cells. Basically, in normal colonic epithelium, luminal butyrate mediates an increase in proliferation in the crypt but under hyperproliferative conditions this is not expected to occur (Holtug et al, 1995). Treatment with selenium greatly restored the normal histoarchitecture in the colonic epithelial cells, with no apparent signs of dysplasia (Ghadi et al., 2009). Although co-treatment with Q revealed inflammatory cells infiltration in lamina properia but was no incidence of dysplasia. Moreover, supplements of Q prior to azoxymethane exposure as carcinogen reduced the incidence of aberrant crypt foci and preneoplastic lesions in rat colon (Volate et al., 2005).

In conclusion, findings of the present study reveal significant role of ROS in pathogenesis of the DMH-induced colon toxicity and initiation of colon cancer. Also, recommended that dietary Q, GNA or the combination of both have a positive beneficial effect against colon cancer progression in mice induced by DMH and manage the promotion of colon tumor.

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