RESEARCH ARTICLE

Amelioration of 1,2 Dimethylhydrazine (DMH) Induced Colon Oxidative Stress, Inflammation and Tumor Promotion Response by Tannic Acid in Wistar Rats

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Abstract

Colon cancer is the third most common malignant neoplasm in the world and it remains an important cause of death, especially in western countries. The toxic environmental pollutant, 1, 2-dimethylhydrazine (DMH), is also a colon-specific carcinogen. Tannic acid (TA) is reported to be effective against various types of chemically induced toxicity and also carcinogenesis. In the present study, we evaluated the chemopreventive efficacy of TA against DMH induced colon toxicity in a rat model. Efficacy of TA against the colon toxicity was evaluated in terms of biochemical estimation of antioxidant enzyme activities, lipid peroxidation, histopathological changes and expression of early molecular markers of inflammation and tumor promotion. DMH treatment induced oxidative stress enzymes ($p<0.001$) and an early inflammatory and tumor promotion response in the colons of Wistar rats. TA treatment prevented deteriorative effects induced by DMH through a protective mechanism that involved reduction of oxidative stress as well as COX-2, i-NOS, PCNA protein expression levels and TNF-α ($p<0.001$) release. It could be concluded from our results that TA markedly protects against chemically induced colon toxicity and acts plausibly by virtue of its antioxidant, anti-inflammatory and antiproliferative activities.

Keywords: DMH - inflammation - oxidative stress - tumor promotion and tannic acid

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Introduction

Colorectal cancer is one of major cause of death in the world, although the causes of this disease are not completely understood. It is the third most common form of malignancy in both men and women (Jemal et al., 2010). Colon carcinogenesis is a multistep process and is thought to arise by the genetic alterations involving a variety of oncogenes and tumor suppressor genes that transform normal colonic epithelium into an invasive carcinoma (Janne and Mayer, 2000). Several environmental factors including lifestyle and perhaps hormones are similarly important in the induction and progression of these tumors. Recent epidemiological data indicate an increased risk of colorectal cancer through consumption of red or processed meat but not white, whereas dietary fibre is protective (Jain et al., 1980; English et al., 2004; Chao et al., 2005; Larsson et al., 2005; Norat et al., 2005).

1, 2 Dimethyl hydrazine (DMH), is a potent colon carcinogen, inducing colorectal tumors in experimental animals (Newell and Heddle, 2004; Saini et al., 2009) and is the most widely used model of chemically induced colon carcinogenesis. DMH induced colon cancer is a multistep process involving a series of pathological alterations, such as formation of aberrant cryptic foci (discrete microscopic lesion; Ionov et al., 1993). Fiala (1975) has already reported that active metabolite of DMH is excreted through bile and is responsible for its carcinogenic effect on colon while passing through digestive tract. DMH is metabolized in liver to form azoxymethane and methylazoxymethanol which is further transported to colon via bile or blood to generate its ultimate carcinogenic metabolite, diazonium ion which elicits an oxidative stress by methylating biomolecules of colonic epithelial cells and leads to promutagenic events as a result of inflammation and tumor promotion.

It is already reported that over-expression of inducible nitric oxide synthase (i-NOS) and COX-2 is associated with colorectal cancer (Umesalma and Sudhandiran, 2010). Over expression of COX-2 has been observed in various premalignant as well as in malignant stage (Williams et al., 1999; Mohan and Epstein, 2003). It has also been found that COX-2 is induced rapidly in response to various growth factors, tumor promoters, hormones, bacterial endotoxins, cytokines and shear stress (Aggarwal and Gehlot, 2009). i-NOS is also known to have integral role in colon toxicity (Eizirik and Pavlovic, 1997). Excessive nitric oxide (NO) production by i-NOS

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can cause damage to DNA, either directly or indirectly by several mechanisms (Hofseth, 2008). It is involved in wide range of physiological and pathological conditions and plays an important role in inflammation and tumor development (Hofseth, 2008). PCNA, a supporting protein to DNA polymerase δ (Fairman, 1990), is a co-factor in DNA synthesis. The synthesis and expression of PCNA is increased in proliferating cells (Bolton et al., 1992). PCNA estimation assay has been considered as the most reliable method to evaluate proliferation in colon tissues (Biasco et al., 1994). Inflammatory cytokines TNF-α play an important role in the initiation and perpetuation of colon cancer (Lee and Lim, 2007; Umesalma and Sudhandiran, 2010). TNF-α is the pro-inflammatory cytokines and several evidences have shown that the expression of TNF-α is dependent on the activation of NF-ϰB which contributes to inflammation (Azzolina et al., 2003).

Naturally occurring phenolics are richly found in edible vegetables, nuts, and fruits and constitute an important part of the human diet (Stoner, 1995). They have been recognized as functionally active molecules, possessing antioxidant, anticancer, antimutagenic properties, as well as exerting protective effects against several other diseases (Ferguson, 2001; Nakamura et al., 2001). Reports from our laboratory have also confirmed antioxidative, anti-genotoxic and anti-tumor promotion role of tannic acid (Sehrawat et al., 2006; Ahmad and Sultana, 2012).

Investigations reporting the protective effects of plant-derived diet based naturally occurring substances on DMH induced colon toxicity are very few. Earlier studies from our laboratory found chrysin and farnesol to be dietary agents that were effective against colon and jejunum carcinogenesis and toxicity of DMH (Khan and Sultana, 2011; Khan et al., 2012). Therefore, in continuation of the search for potential modulators of DMH-induced colon damage, we have examined the effects of tannic acid on experimentally induced DMH-toxicity in Wistar rats. Furthermore, the effect of tannic acid in DMH-induced tumor promotion and inflammatory response was also evaluated.

Materials and Methods

Chemicals

Reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate reduced (NADPH, ethylene diamine tetra-acetic acid (EDTA), nicotinamide adenine dinucleotide reduced (NADH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), bovine serum albumin (BSA), 1,2-dithio-bis-nitrobenzoic acid (DTNB), Tannic acid, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-Dimethylhydrazine (DMH), glutathione reductase were obtained from Sigma (Chemical Co.,St Louis, MO), ferric nitrate, ammonium thiocyanate, hydrogen peroxide, magnesium chloride, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate and sodium hydroxide were purchased from E. Merck Limited, India. Primary antibodies were rabbit anti-COX-2 (dilution 1:200, Santa Cruz, USA), rabbit anti-iNOS (1:500, Jackson ImmunoResearch laboratories, INC.) and PCNA (dilution 1:300,).

Animals and Treatment schedule

The experiments were carried out using male Albino Wistar rats, 120-150 g, obtained from the Central Animal House of Hamdard University, New Delhi, India. 42 animals were randomly divided into seven groups of six animals each. The rats were kept in polypropylene cages and were kept in room maintained at 25±2˚C with a 12 h light/dark cycle. They were given free access to diet and water. Figure 1 shows the treatment protocol followed; the rats of Group I control group received distilled water orally three times a week for whole study. Group II received DMH subcutaneous at the dose of 20 mg/kg body weight once a week. Group III (dose 1 of TA) received TA orally at the dose of 50 mg/kg body weight three times a week for 14 consecutive weeks. Group IV and V (dose 2 of TA) received TA at the dose of 100 mg/kg body weight three times a week for 14 consecutive weeks. Group VI (dose 1 of TA) received orally at the dose of 50 mg/kg body weight three times a week, from sixth week till end of study. Group VII (dose 2 of TA) received orally at the dose of 100 mg/kg body weight three times a week from sixth week till end of study. Group II, III, IV,VI and VII rats were given a five time injection of DMH (dissolved in 1mM EDTA solution pH a adjusted to 6.5 with 1mM NaOH) at the dose 20 mg/kg body weight, subcutaneously in the groin start after one week from administration of treatment TA. Group I and V rats also received subcutaneous injection of 1mM EDTA solution, (pH adjusted to 6.5 with 1mM NaOH), which was used as the vehicle for DMH. All the rats were anaesthetized with mild anaesthesia and sacrificed by cervical dislocation after 24 hr of the DMH injection. The doses of tannic acid were selected on the basis preliminary studies performed in our laboratory and also on the basis of available literature (Sehrawat et al., 2006; Ahmad and Sultana, 2012). Our study was approved by the Institutional Animal Ethics Committee (IAEC) of the university with registration no 599.

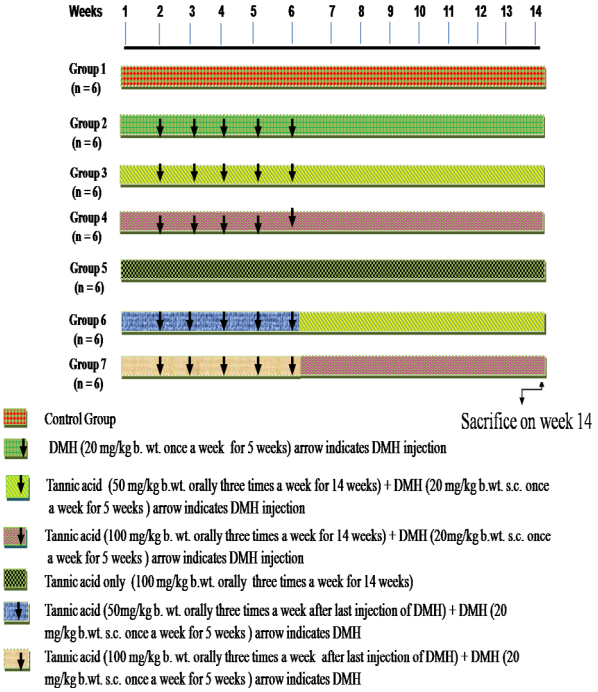

Figure 1. Treatment Protocol for Different Groups.

Post-mitochondrial supernatant (PMS) preparation and estimation of different parameters

Colons were removed quickly, cleaned free of irrelevant material and immediately perfused with ice-cold saline (0.85% sodium chloride). The colons (10% w/v) were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) using a Potter Elvehjen homogenizer. The homogenate was filtered through muslin cloth, and were centrifuged at 3000 rpm for 10 min at 4°C by Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 rpm for 20 min at 4°C to obtain PMS, which was used as a source of various enzymes (Rehman and Sultana, 2011).

Measurement of reduced glutathione level (GSH)

The GSH content in colon was determined by the method of Jollow et al. (1974) in which 1.0 ml of PMS fraction (10%) was mixed with 1.0 ml of sulphosalicylic acid (4%). The samples were incubated at 4°C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4°C. The assay mixture contained 0.4 ml filtered aliquot, 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (10mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on spectrophotometer (Milton Roy Model-21 D). The GSH content was calculated as nmol of DTNB conjugate formed/g tissue using molar extinction coefficient of $13.6\times103\ M^{-1}\ cm^{-1}$.

Measurement of malondialdehyde (MDA)

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was estimated by the method described by Ohkawa et al. (1979). To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% Sodium dodecylsulphate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA were added. The mixture was made upto 4.0 ml with distilled water and then heated in a boiling water bath at 95°C for 60 min. After cooling, 1.0 ml water and 5.0 ml n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 600×g for 10 min, the pink coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. The results were expressed as the nmol MDA formed/min/gram tissue by using a molar extinction coefficient of $1.56\times10^5\ M^{-1}\ cm^{-1}$.

Measurement of glutathione peroxidase (GPx) activity

The GPx activity was calculated by the method of Mohandas et al. (1984). A total of 2 ml volume consisted of 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 1.44 ml phosphate buffer (0.1M, pH 7.4), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml reduced glutathione (1 mM), 0.1 ml NADPH (0.2 mM) and 0.01 ml H_2O_2 (0.25 mM) and 0.1ml 10% PMS. The depletion of NADPH at 340 nm was recorded at 25°C. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein with the molar extinction coefficient of $6.22X10^3\ M^{-1}\ cm^{-1}$.

Measurement of Glutathione reductase (GR) activity

The GR activity was measured by the method of Carlberg et al. (1975). The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1.0 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml of 10% PMS in a total volume of 2.0 ml. The enzyme activity was assessed at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22\times10^3\ M^{-1}\ cm^{-1}$.

Measurement of glutathione-S-transferase (GST) activity

The GST activity was measured by the method of Habig et al. (1974). The reaction mixture consisted of 2.4 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1.0 mM), 0.2 ml CDNB (1.0 mM) and 0.2 ml of cytosolic fraction in a total volume of 3.0 ml. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6\times10^3\ M^{-1}\ cm^{-1}$.

Measurement of catalase activity

The catalase activity was measured by the method of Claiborne et al. (1985). In brief, the assay mixture consisted of 2.0 ml phosphate buffer (0.1 M, pH 7.4), 0.95 ml hydrogen peroxide (0.019 M) and 0.05 ml of PMS (10%) in a final volume of 3.0 ml. Changes in absorbance we are recorded at 240 nm. The catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

Measurement of SOD activity

The SOD activity was measured by the method of Marklund, (1974). The reaction mixture consisted of 2.875 ml Tris–HCl buffer (50 mM, pH 8.5), pyrogallol (24 mM in 10mM HCl) and 100 µl PMS in a total volume of 3 ml. The enzyme activity was measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

Protein estimation

The protein concentration in all samples was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Assay for TNF-α level

TNF-α protein level was measured by enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, Inc., San Diego., USA). Analysis was performed according to the manufacturer's instruction.

Histopathology

The colon were excised, flushed with saline, cut open longitudinally along the main axis, and then again washed with saline. These colonic sections fixed in 10% buffered formalin for at least 24 h and after fixation, the specimens were dehydrated in ascending grades of ethanol, cleared in benzene, and embedded in paraffin wax. Blocks were made and 5µm thick sections were cut from the distal colon. The paraffin embedded colonic tissue sections

were deparaffinized using xylene and ethanol. The slides were washed with phosphate buffered saline (PBS) and permeabilized with permeabilization solution (0.1M citrate, 0.1% TritonX-100). These sections stained with haematoxylin and eosin and were observed under light microscope at 10X and 40X magnifications to investigate the histoarchitecture of colonic mucosa.

Immunohistochemical staining for detection of (i-NOS, COX-2, PCNA)

Section of 4µm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-L-lysine coated microscopic slides. The protocol followed it was according to (Ahmad et al., 2011) Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water followed by antigen retrieval in sodium citrate buffer (10mM, pH 6.0). The slides were then allowed to cool for 15 minutes and washed 3 times with tris-buffered saline (TBS) for 5 minutes each. Slides the incubated in 3% H_2O_2 in methanol for 10 min to reduce the endogenous peroxidase activity and then subject to Ultra block (UltraVision Plus Detection System, Thermo Scientific) for 10 min to block non-specific binding. After rinsing the sections in TBS, the slides were incubated overnight at 4°C with primary antibody inside humidified chamber and then were washed in TBS. The sections were incubated with biotinylated goat anti-polyvalent secondary antibody (UltraVision Plus Detection System, Thermo Scientific) for 20 min and then were rinsed in TBS. The sections were again incubated with streptavidin peroxidase plus (UltraVision Plus Detection System, Thermo Scientific) for 30 min. The sections were washed in TBS and developed with 3, 3'-Diaminobenzidine (DAB) solution (UltraVision Plus Detection System, Thermo Scientific) until sections become brown. Counterstained the sections with Mayer's haematoxylin, mounted by using mounting media and then visualized under the light microscope (Olympus BX51). Primary antibody of rabbit ant dilution was used.

Results

Effect of TA on the level of colonic GSH

The level of GSH was depleted significantly ($p<0.001$) in DMH treated group (Group II) as compared to control group (Group I). TA pre-treatment showed a significant increase in the level of GSH in Group III ($p<0.01$), Group IV ($p<0.001$), and post- treatment Group VII ($p<0.01$) when compared with group II. Post-treatment Group VI exhibited no significant changes in the level of GSH as compared to control Group II (Table 1).

Effect of TA on the activities of glutathione dependent enzymes in colonic tissue

The activities of GPx, GR and GST decreased significantly ($p<0.001$) in Group II as compared to Group I. TA pre-treatment at the dose of (50 mg/kg b. wt). Significantly increased the activities of GPx ($p<0.01$), GST ($p<0.001$), and GR ($p<0.001$) in Group III as compared to Group II. Higher dose of TA (100 mg/kg b.wt) also showed increase in the activities of GPx ($p<0.001$), GST ($p<0.001$), and GR ($p<0.001$) in Group IV as compared to Group II. TA post-treatment at the dose of (50 mg/kg b. wt) significantly increased the activities of GPx ($p<0.01$) and GST ($p<0.001$), in Group VI as compared to Group II. However, the activities of GR in group VI did not change significantly as compared to Group II. TA post-treatment at dose (100 mg/kg b. wt) also showed increased the activities of GPx ($p<0.001$), GST ($p<0.001$), and GR ($p<0.001$) in Group IV as compared

Table 1. Effect of Tannic and DMH on the Glutathione Peroxidase (GPX), Glutathione S-transferase (GST), Glutathione Reductase and Reduced Glutathione (GSH)

Treatment Groups	GR[1]	GST[2]	GPX[3]	GSH[4]
Vehicle treatment control	455.9±18.52I	369.0±14.28	629.5±9.56	0.037±0.0052
II DMH Group	231.5±23.58[a]	103.0±8.18[a]	286.2±16.22[a]	0.010±0.0028[a]
III Pre-treatment TA D1 + DMH	356.5±23.58[c]	284.6±16.19[d]	540.1±31.14[d]	0.0081±0.0081[c]
IV Pre-treatment TA D2 +DMH	442.8±16.03[d]	387.3±7.03[d]	642.3±27.73[d]	0.029±0.0049[d]
V TA D2 only	429.4±30.7	379.2±16.02	642.4±15.50	0.037±0.0064
VI Post- treatment TA D1+DMH	274.2±10.84[e]	207.2±7.03[d]	398.5±15.22[c]	0.014±0.0028[c]
VII Post- treatment TA D2+DMH	385.6±27.05[d]	269.3±16.02[d]	505.7±19.9[d]	0.024±0.0058[c]

Table 2. Effects of Tannic Acid and DMH on the Catalase (CAT), Superoxide Dismutase (SOD) and Lipid Peroxidation (LPO)

Treatment Groups	Catalase[5]	SOD[6]	LPO[7]
Vehicle treatment control	25.61±0.87	15±1.2	6.06 ±0.7
II DMH Group	14.88±0.26**	11±2.6**	15.70 ±0.98[a]
III Pre-treatment TA D1 + DMH	20.41±2.91[e]	12±1.2[e]	8.559±0.85[d]
IV Pre-treatment TA D2 +DMH	25.57±3.14[c]	14±0.6[c]	6.80 ±1.03[d]
V TA D2 only	25.61±1.76	15±2.1	5.40 ±1.79
VI Post treatment TA D1+DMH	20.89±0.53[c]	11±1.9[e]	10.87 ±0.33[c]
VII Post-treatment TA D2+DMH	24.69±1.47[b]	13±1.7[b]	6.79 ±0.65[d]

*Results represent mean±SE of six animals per group; Results obtained are significantly different from Control group ([a]$P<0.001$); Results obtained are significantly different from DMH treated group ([b]$P<0.05$, [c]$P<0.01$, [d]$P<0001$, [e]ns < not significant); TA: Tannic acid; D1=50 mg/kg/b wt; D2=100mg/kg/b wt; [1]nmoles NDPH oxidized/min/mg protein; [2]nmoles CDNB conjugate formed/min/mg protein; [3]nmoles of NADPH oxidize/min/mg protein; [4]nmoles of DTNB conjugate formed/min/mg protein; [5]nmoles of H_20_2 consumed/min /mg protein; [6]Unit per mg/ protein; [7]nmoles of MDA formed /g tissue).

to Group II (Table 1).

Effect of TA on the activities of colonic antioxidant enzymes

DMH treatment caused significant decrease in the activities of SOD ($p<0.01$) and Catalase ($p<0.001$) in Group II as compared to Group I. TA pre-treatment at dose (50 mg/kg b. wt) did not show any significant change in SOD and catalase activities in Group III as compared to Group II. TA pre-treatment at dose (100 mg/kg b. wt) showed significant increase in the activities of SOD ($p<0.01$), and catalase ($p<0.01$) in Group IV as compared to Group II. TA post-treatment (50 mg/kg b. wt) showed significant increase in the activities of SOD ($p<0.01$). TA post-treatment at dose (100 mg/kg b. wt) showed significant increase in the activities of SOD ($p<0.01$) and catalase ($p<0.01$) in Group VI as compared to Group II. Post-treatment dose of only TA (50 mg/kg b. wt) did not change significantly of catalase activities in Group VI as compared to Group II. Also TA Post-treatment at dose of (100 mg/kg b.wt) showed significant increase in the activities of SOD ($p<0.05$) and catalase ($p<0.05$) in Group VII as compared to Group II (Table 2).

Effect of TA on the level of MDA in colonic tissue

The level of MDA were remarkably increased ($p<0.001$) in Group II as compared to Group I. TA pre-treatment at dose (50 mg/kg b.wt) significantly ($p<0.001$) decreased the level of MDA in Group III as compared to Group II. Pre-treatment of higher dose TA (100 mg/kg b.wt) also significantly ($p<0.001$) decreased the level of MDA in Group IV as compared to Group II. TA post-treatment at dose (50 mg/kg b.wt) significantly ($p<0.01$) decreased the level of MDA in Group VI as compared to Group II. Post-treatment of higher dose TA (100 mg/kg b.wt) also significantly ($p<0.001$) decreased the level of MDA in Group VII as compared to Group II (Table 2).

Effect of TA on colon tissues proinflammatory cytokine (TNF-α)

We have assessed the effect TA on DMH induced abnormal increase in TNF-α level (Figure 2). We found that there was a significant increase in the level of proinflammatory cytokines in DMH group II compared with control group I ($p<0.001$). Pre-treatment with TA at dose (50 mg/kg b. wt) significantly inhibit their abnormal increase in the group III when compared with the only DMH treated group II ($p<0.01$). Pre-treatment with higher dose of TA (100 mg/kg b. wt) significantly inhibit their increase in TNF-α in the group IV when compared with the only DMH treated group II ($p<0.001$). Post-treatment with TA at dose (50 mg/kg b. wt) showed no significant difference in levels of this proinflammatory cytokine between group VI and group II. Post-treatment with higher dose of TA (100 mg/kg b. wt) significantly inhibit their increase in TNF-α in the group VII when compared with the only DMH treated group II ($p<0.05$) There was no significant difference in levels of this proinflammatory cytokine between group I and group V.

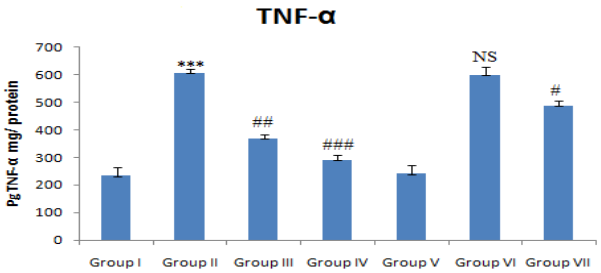

Figure 2. Effect of TA on DMH Induced Level of TNF-α in Colon Tissue. In DMH-treated (group II), the level of TNF-α was increased significantly (***$p<0.001$) as compared to control group. While treatment with tannic acid significantly attenuated the level of TNF-α in Group III (##$p<0.01$), IV (###$p<0.001$) and VII (#$p<0.05$) as compared to Group II, as well as no significant difference in the level of TNF-α in Group VI as compared to Group II. There was no significant difference in the level of TNF-α in Group V as compared to Group I.

Effect of TA on DMH-induced over expression of COX-2 and i-NOS

In Group II (DMH only) the COX-2 and i-NOS proteins showed more positive staining compared to group I (Control). In pre-treatment group III TA at dose (50 mg/kg b.wt) the COX-2 and i-NOS expression appeared slightly decrease in colon tissue compared to group II (DMH only). In pre-treatment Group IV TA at dose (100 mg/kg b. wt) showed substantial decrease in COX-2 and i-NOS protein expression in colonic tissue as compared to group II (DMH only). No significant difference was observed in staining pattern in group V as compared group I (control). In case of post-treatment in group VI, TA at dose (50 mg/kg b.wt) showed slight decrease in COX-2 and i-NOS expression. In post-treatment in group VII TA at dose (100 mg/kg b.wt) showed considerable decrease in COX-2 and i-NOS expression as compared to group II (DMH only) but decrease was not as effective as in case of pre-treatment (Figure 3 and 4).

Effect of TA on DMH-induced increase in PCNA expression in colonic tissue

In Group II (DMH only) the PCNA showed increased number of positive nuclei staining compared to group I (vehicle). In pre-treatment group III TA at dose (50 mg/kg b.wt) the PCNA positive staining appeared slightly decreased in colon tissue compared to group II (DMH only). Pre-treatment group IV higher dose of TA (100 mg/kg b.wt) showed substantial decrease in the number of PCNA positive nuclei in colonic tissue as compared to group II (DMH only). There was no significant difference in staining pattern in group V as compared to group I (vehicle). In case of post-treatment TA at dose (50 mg/kg b.wt) there was slight decrease in the number of staining of PCNA positive nuclei. In post-treatment group VII higher dose of TA (100 mg/kg b.wt) showed considerable decrease in the nuclei staining of PCNA compared to group II (DMH only) but decrease was not as effective as in case of pre-treatment (Figure 5).

Histopathological findings

Histological sections of control group (group I) exhibited normal histoarchitecture of the colon. In DMH-treated group (group II), irregular glandular

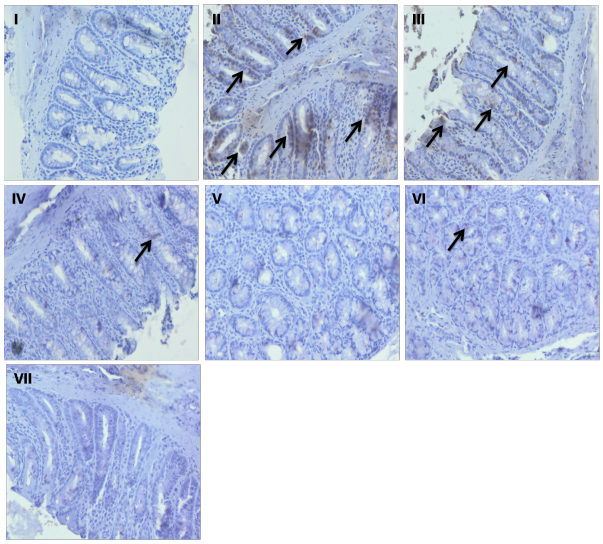

Figure 3. Effect of TA Treatment on DMH Induced Colon Expression of COX-2 in Colon Tissue. Representative photomicrographs (magnification x40). Brown color indicates COX-2 specific staining and blue color indicates haematoxylin staining.DMH treated group (Group II) shows more COX-2 immunopositive staining as compared with vehicle treated group (Group I).TA pre-treatment (Group III & IV) reduces COX-2 expression as compared to Group II. However there was no significant difference in the COX-2 immunostaining in Group V as compared to Group I. Post-treatment groups (Group VI & VII) also showed lesser COX-2 positive immunostaining as compared to Group II. Black arrows indicate region of positive immunostaining reactivity.

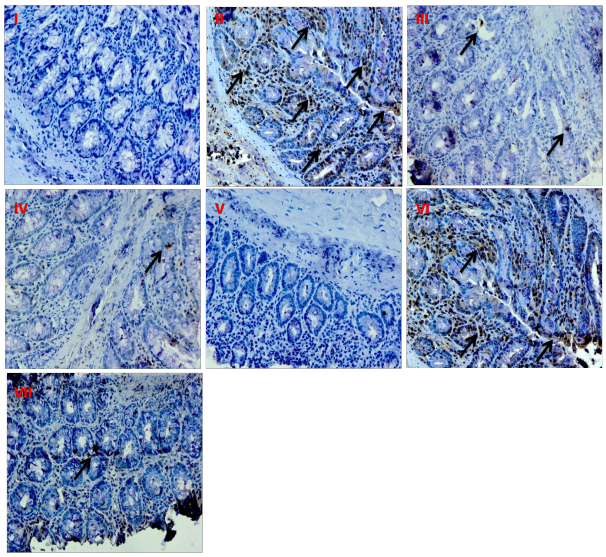

Figure 4. Effect of TA on DMH Induced Colon Expression of i-NOS in Colon Tissue. Representative photomicrographs (magnification x40). Brown color indicates i-NOS specific staining and blue color indicates haematoxylin staining. DMH treated group (Group II) shows more intense i-NOS immunopositive staining as compared with vehicle treated group (Group I).TA pre-treatment (Group III & IV) reduces i-NOS expression as compared to Group II. However there was no significant difference in i-NOS immunostaining in Group V as compared to Group I. Post-treatment groups (Group VI & VII) also showed lesser i-NOS positive immunostaining as compared to Group II. Pre-treatment groups (III & IV) showing TA to be more potent inhibitor of i-NOS as compared to post-treatment groups (Group VI & VII). Black arrows indicate region of positive immunostaining reactivity.

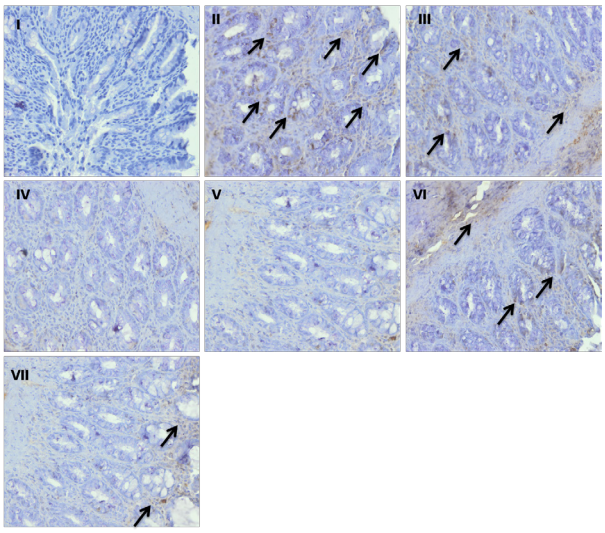

Figure 5. Effect of TA on DMH Induced Expression of PCNA in Colon Tissue. Representative photomicrographs (magnification x40). Brown color indicates PCNA specific staining and blue color indicates haematoxylin staining. DMH treated group (Group II) shows more PCNA positive cells as compared with vehicle treated group (Group I).TA pre-treatment (Group III & IV) inhibits PCNA expression as compared to Group II. However there was no significant difference in PCNA immunostaining in Group V as compared to Group I. Post-treatment groups (Group VI & VII) also showed lesser PCNA positive cells as compared to Group II. Pre-treatment groups (III & IV) showing TA to be more potent inhibitor of cellular proliferation PCNA as compared to post-treatment groups (Group VI & VII). Black arrows represent PCNA positive cell.

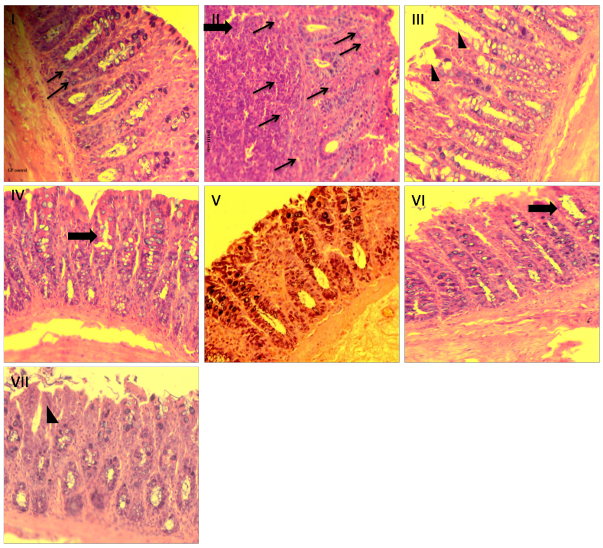

Figure 6. Effect of TA on DMH Induced Changes in Colonic Histo-architecture. Group I (Vehicle group) shows the normal histoarchitecture of the colon with normal mucosal glandular structure with mild inflammatory cells infiltration. Group II shows massive inflammatory cells infiltration(arrows) with crypt abscess formation (bold arrows) Group III shows crypt ablation(arrowhead) with less inflammatory cells infiltration as compared to Group IV shows crypt abscess formation with mild inflammatory cells infiltration. Group V shows normal histoarchitecture of the colon with normal mucosal glandular structure with mild inflammatory cells infiltration as shows in GroupI. Group VI shows crypt abscess formation (bold arrows) with normal mucosal glandular structure and normal histoarchitecture of the colon. Group VII shows crypt ablation (arrowhead) with mild inflammatory cell infiltration.

structure, regional destruction of the mucosa with severe inflammatory cells infiltration in mucosal and submucosal layers as well as submucosal edema were examined and mid colon of a prolonged reactivation rat showing large lymphoid follicles invading the lamina propria, causing loss of mucosal architecture. In group III (pre-treatment) and group IV (pre-treatment), histological sections showed that TA protected the mucosal from damage and there was notable decrease in the inflammatory cells permeation and submucosal edema. In Group V tissue sections displayed normal architecture as similar to that of Group I (vehicle treated). In group VI (pot-treatment) and group VII (post-treatment), histological region in TA treatment showed slight decrease in mucosal damage, inflammatory cells permeation and submucosal edema, but the protection was not as effective as in pre-treatment (Figure 6).

Discussion

Chemoprevention is the use of plant based nontoxic substance, including many dietary agents, to avert or reverse the process of cancer development. Polyphenols, which are among the most common naturally occurring anti-oxidants in fruits and vegetables, have been proposed as primary chemopreventive agents; several of these chemopreventive agents are suggested to confer protection against a wide range of cancers including colon cancer (Rajamanickam and Agarwal, 2008; Kang et al., 2011). Tannic acid is reported to be effective against various chemically induced toxicities and also carcinogenesis (Athar et al., 1989; Gali et al., 1992; Horikawa et al., 1994; Nepka et al., 1999; Chen and Chung, 2000; Ferguson, 2001; Ahmad et al., 2011; Ahmad and Sultana, 2012). In present study we showed that pretreatment as well as post treatment with TA resulted in the protection against DMH-induced colon toxicity by amelioration of oxidative stress and inflammatory damage.

We have previously demonstrated the role of oxidative stress in colon toxicity induced by several toxicants such as DMH and Cisplatin (Khan and Sultana, 2011). DMH is a procarcinogen and 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 stages and lipid peroxidation. Lipid peroxidation or MDA formation is one of the important and relevant markers of oxidative damage and elevated level of lipid peroxidation product (MDA) has been found after treatment with DMH (Dudeja and Brasitus, 1990; Kawanishi and Yamamoto, 1991; Sengottuvelan et al., 2006). Consistent with previous reports our results also showed remarkable increase in the level MDA after DMH treatment. TA pre-treatment and post treatment significantly attenuated elevated levels of MDA, from the results are in agreement with recently published report from our laboratory where tannic acid shown to be effective against Cisplatin induced renal toxicity (Ahmad and Sultan, 2012).

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). GSH and its oxidized counterpart represent a major redox buffer system of the cell. GSH can act either as a non-enzymatic antioxidant by direct interaction of -SH group with ROS or it can be implicated in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme. The GSH levels in rats treated with DMH showed significant decrease in our study and previous studies (Rajeshkumar and Kuttan, 2003; Khan et al., 2012), and the treatment with TA restored the normal levels of GSH indicating protective efficacy of TA (Ahmad and Sultana, 2012).

DMH treatment generates free radicals in colonic tissue and their level is controlled by GSH and other enzymatic antioxidants like (SOD, Catalase, GPx, GR etc.) by scavenging the free radicals. Thus DMH treatment decreases level of antioxidant enzymes (Rajeshkumar and Kuttan, 2003; Sengottuvelan et al., 2006). Both TA pre-treatment and post-treatment significantly brought these levels back to normal. Protective efficacy of tannic acid in DMH induced oxidative stress can be possibly due to its ability to induce antioxidant enzymes like GR, GST.GPX etc. as previously reported from our laboratory and by others too (Tsujiiet al., 1998; Gülçin et al., 2010; Ahmad and Sultana, 2012; Baskar et al., 2012).

Oxidative stress and inflammation play important role and are documented to influence tumor initiation and promotion (Bickers and Athar, 2006). COX-2 an important inflammatory marker is induced during inflammation, up-regulated in adenomas and over-expressed in colon cancer (Eberhart et al., 1994; Sano et al., 1995). COX-2 has been shown to plays a key role in colon toxicity and polyp formation and its inhibition has proven to be an important strategy for chemopreventive treatment of colon related disorders (Taketo, 1998). We observed that DMH-treated rats showed increased expression of COX-2 and pre-treatment of tannic acid efficiently inhibited the expression of COX-2 and post treatment also inhibited over expression of COX-2 but not as effectively as in case of pre-treatment. i-NOS, another inflammatory marker has been shown to be up-regulated in DMH-induced colon tumrogenesis (Jenkins et al., 1995; Samanta et al., 2008), and its inhibition is yet another important approach to prevent colon cancer (Rao et al., 1999). We also observed increased pattern of i-NOS expression in DMH treated group and tannic acid inhibited this over-expression of i-NOS in pre-treatment and post-treatment condition.

In previous studies, ellagic acid efficiently reduced the expressions of TNF-α in DMH-induced colonic tumors and thus exhibited its anti-inflammatory activity (Chao et al., 2009).

TNF-α is the pro-inflammatory cytokines and several evidences have shown that the expression of TNF-α is dependent on the activation of NF-ϰB which is one of the key inflammatory mediators (Azzolina et al., 2003). Therefore, targeting TNF-α is an effective approach for the treatment of colon cancer (Van Grevenstein et al., 2007). Our data suggest that TA effectively suppress the production of these cytokines which implicates its possible role in chemoprevention of colon cancer.

PCNA is non-histone nuclear acidic protein expressed in the nuclei of proliferating cells during G1 and S phase of cell cycle (Bravo, 1986; Bravo et al., 1987; Jaskulski

et al., 1988). It is already reported to be an important biomarker in gastrointestinal cancer (Kelman et al., 1999; Nagendraprabhu and Sudhandiran, 2011). In this study, DMH administered rats showed amplified expression of PCNA in the colon thereby indicating the hyper-proliferative activity of colon cells. Over expression of PCNA protein was directly related to increased proliferation, because it plays an important role in nucleic acid metabolism as a part of the replication (Kelman et al., 1999). Decreased expression of this important proliferative marker was clearly noted upon Tannic acid pre-treatment and post-treatment.

In this study findings of antioxidative, anti-inflammatory and anti-tumor-promoting potential of Tannic acid were supported by histological data. We observed intense inflammation in mucosal and sub mucosal layer with extensive mucosal layer destruction in DMH treated groups, sub-mucosal edema can also be seen at some places (Khan et al., 2012). Tannic acid treatment suppressed massive inflammatory response in both pre-treatment and post-treatment conditions.

In conclusion the finding of the present study reveal significant role of ROS, inflammatory pathway proteins like COX-2, i-NOS and early tumor promotion marker PCNA in pathogenesis of the DMH-induced colon toxicity and early neoplastic transitions. It further demonstrates that abrogation of inflammatory and tumor promotion response by naturally occurring polyphenolic antioxidants could be effective strategy for prophylaxis of DMH-induced colon toxicity. Therefore Tannic acid may prevent the tumor promoting property of DMH through antioxidant and anti-inflammatory properties and might be useful clinically after further molecular chemopreventive studies.

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