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Modulatory influence of dietary resveratrol during different phases of 1,2-dimethylhydrazine induced mucosal lipid-peroxidation, antioxidant status and aberrant crypt foci development in rat colon carcinogenesis

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Abstract

To shed light on the association of lipid peroxidation and antioxidant status with the development of aberrant crypt foci (ACF), we studied the modulatory influence of resveratrol, supplemented in three dietary regimens (initiation, post-initiation and entire period) on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis. Rats were administered DMH (20 mg/kg body weight, s.c.) for 15 weeks and were supplemented with resveratrol (8 mg/kg body weight, p.o. everyday) in three dietary regimens. Intestines and colons were analyzed for the levels of diene conjugates (DC), lipid hydroperoxides (LOOHs) and thiobarbituric acid reactive substances (TBARS). Enzymic antioxidants (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX; glutathione *S*-transferase, GST; and glutathione reductase, GR) and non-enzymic reserve (reduced glutathione, GSH; ascorbate; and α-tocopherol) were also assessed in the intestine and colon. Unsupplemented DMH exposed rats showed significantly decreased levels/activities of tissue DC, LOOHs, TBARS, SOD, CAT, GSH, GR and significantly elevated ($P<0.05$) GPX, GST, α-tocopherol and ascorbate as compared to control rats. Resveratrol supplementation during the entire period of the study resulted in significant ($P<0.01$) modulation of lipid peroxidation markers and antioxidants status, which were paralleled with ACF suppression, as compared to DMH-alone treated rats. These results indicate that resveratrol effectively inhibits DMH-induced ACF and colonic tumor development.

Keywords: Antioxidants; Colon carcinogenesis; Oxidative stress; Resveratrol

1. Introduction

Cancer of the colon is one of the most common cancers in developed countries and its prevention is of great interest throughout the world [1]. During the early stages, neoplastic development by environmental genotoxic and non-genotoxic carcinogens act predominantly by triggering the free radical mediated damage [2]. The carcinogen, 1,2-dimethylhydrazine (DMH) is an alkylating agent, the metabolic events of which are believed to occur in the liver with the formation of active intermediates such as azoxymethane and methylazoxymethanol, that are subsequently transported to the colon via bile or the blood [3]. The decomposition of methylazoxymethanol results in the formation of methyldiazonium ions, which generate reactive carbonium ions capable of methylating DNA, RNA, or protein of colonic epithelial cells [4]. Moreover, previous reports have pointed out the tendency of DMH to produce free radicals in blood, liver and large bowel [5] of experimental models.

An increase in the reactive oxygen metabolites (superoxide radical and hydrogen peroxide, precursors of a number of oxygen-derived radicals including hydroxyl radicals) in tumor cells are noted in the early stages, and the overproduction and/or the inability to destroy them may result in severe damage to cell molecules and structures. During carcinogenesis, reactive

Abbreviations: ACF, Aberrant crypt foci; CAT, Catalase; DC, Diene conjugates; DMH, 1,2-dimethylhydrazine; GLUTs, Glucose transporter; GPX, Glutathione peroxidase; GR, Glutathione reductase; GSH, Reduced glutathione; GST, Glutathione *S*-transferase; LOOH, Lipid hydroperoxides; PUFA, Polyunsaturated fatty acids; SOD, Superoxide dismutase; TBARS, Thiobarbituric acid reactive substances

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oxygen radicals may damage specific genes that control the growth and differentiation of cells [6]. Recently, a general scheme has been proposed to describe the role of oxy-radicals during the initiation and promotion stages of carcinogenesis, which leads to decreased PUFA content in the tumor subcellular membranes. This in turn would diminish the lipid peroxidizability of these membranes, reducing the production of lipid hydroperoxides in later stages of carcinogenesis and may confer an advantage to overwhelm oxidative stress [7].

Cells are equipped with an impressive repertoire of antioxidant enzymes as well as non-enzymic small antioxidant molecules [8]. Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9) are considered to be the primary antioxidant enzymes, since they are involved in the direct elimination of reactive oxygen species. Glutathione *S*-transferase (GST, EC 2.5.1.18) and glutathione reductase (GR, EC 1.6.4.2) are redox-regulating enzymes, which help in the detoxification of reactive oxygen species by decreasing peroxide levels or by maintaining a steady supply of metabolic intermediates like glutathione, for the primary defense enzymes. The non-enzymic small molecular antioxidants including sulphydryl compounds such as glutathione (GSH), ascorbate and α-tocopherol also play a significant role in reducing the oxidative stress. Since these antioxidants work co-operatively, a change in any one of them may break the equilibrium and cause cell damage leading to malignancy [9].

Aberrant crypt foci (ACF) are putative preneoplastic lesions of colonic neoplasia in rodents and humans [10]. During the process of colon carcinogenesis, ACF appear in the early stages and sequentially develop into polyps, adenomas and eventually into carcinomas [11].

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenolic compound enriched in grapes and red wine, has received wide attention lately due to its ability to serve as an effective antioxidative agent [12]. It inhibits cellular events associated with tumor initiation, promotion/progression [13]. Recently, resveratrol was found to possess cancer preventive activity in several animal cancer models, such as by blocking the development of preneoplastic lesions in carcinogen-treated mouse mammary glands and two-stage model of skin cancer [14]. Although anti-carcinogenic function of resveratrol has been well established, the mechanism by which resveratrol exerts its chemopreventive effects remain largely unknown. The colon cancer model was chosen because it is the unique organ that is exposed to both circulatory and luminal dietary resveratrol.

The primary objective of the study was to assess the influence of resveratrol on DMH-induced colon carcinogenesis by correlating lipid peroxidation and antioxidant status with the modulation of ACF.

2. Materials and methods

2.1. Animals

Five-week-old, male Wistar rats were purchased from Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University, quarantined for 1 week and allocated randomly to experimental or control groups. Animals were maintained as per the principles and guidelines of the Ethical Committee of Animal Care of Annamalai University in accordance with the Indian National Law on Animal Care and Use (Reg. No. 160/1999/CPCSEA). The animals were housed four per cage in a specific pathogen-free animal room under controlled conditions of a 12 h light/12 h dark cycle, with temperature of 22±1 °C and relative humidity of 50±10% till the end of 30 weeks.

2.2. Chemicals

1,2-Dimethylhydrazine, *trans*-resveratrol, 1,1,3,3-tetramethoxypropane, NBT (nitroblue tetrazolium), GSH (reduced glutathione), DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) CDNB (1′-chloro-2,4-dinitrobenzene), NADPH (nicotinamide adenine dinucleotide phosphate) and BSA (bovine serum albumin) were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade and obtained from Hi-Media Laboratories, Mumbai.

2.3. Experimental design

Rats in group 1 received no treatment and served as the untreated control. Group 2 animals received resveratrol by intragastric intubation daily at the dose of 8 mg/kg body weight suspended in 1% carboxymethylcellulose (CMC). Rats in groups 3–6 received DMH injections (20 mg/kg body weight in 1 mM EDTA, the pH adjusted to 6.5 with 1 mM NaOH and used immediately). Injections were given once a week subcutaneously for the first 15 weeks. Group 4 (initiation) rats received resveratrol as in group 2 starting 1 week before DMH injections and continued till 1 week after the final exposure of DMH (DMH+RES (I)). Group 5 (post-initiation) rats received resveratrol as in group 2 starting 2 days after the cessation of DMH injections and continued till the end of 30 weeks (DMH+RES (PI)). Group 6 (entire-period) animals received resveratrol as in group 2 starting on the day of DMH injection and continued till the end of experimental period (DMH+RES (EP)). The experiment was terminated at the end of 30 weeks. The experimental protocol is clearly represented in Fig. 1.

2.4. ACF and tumor enumeration

After 30 weeks, animals were sacrificed; the colons were removed, flushed with saline, slit open longitudinally from the cecum to anus. For enumerating the ACF, tissues were fixed with 10% buffered formalin for 24 h and stained with 0.2% methylene blue as described by Bird and Good [15]. The colon was examined grossly and histologically for tumor types. The tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Fixed tissues were cut into serial sections of 3 μm wide strips and stained with H&E for histological examination. Benign tumors were defined as those adenomatous polyps, which showed no evidence of invasion through the muscularis mucosa. Tumors were considered to be malignant (adenomacarcinoma) only when there was a clear-cut evidence of tumor invasion through the muscularis mucosa. All sections were examined without knowledge of their group of origin.

2.5. Preparation of tissue homogenate

The mucosal scrapings and excised tumor tissues were homogenized in 3 volumes (w/v) of phosphate buffer (0.1 M, pH 7.4), centrifuged at 12,000×g for 20 min at 4 °C and the supernatant was used for assays.

2.6. Determination of lipid peroxidation products

Tissue diene conjugates (DC) were analyzed by the method of Recknagel and Glende [16]. Lipids were extracted from tissue samples by using chloroform/methanol (2:1) mixture, dried under nitrogen atmosphere, dissolved in cyclohexane and measured spectrophotometrically at 232 nm (Perklin Elmer UV/VIS Spectrometer). The results were expressed as mmol/mg tissue.

For the estimation of lipid hydroperoxides (LOOH), 0.1 ml of tissue homogenates (supernatant) were treated with 0.9 ml Fox reagent (100 μM xylenol orange, 4 mM butylated hydroxytoluene, 25 mM sulphuric acid and 250 μM ammonium ferrous sulphate) and was incubated at 37 °C for 30 min. The

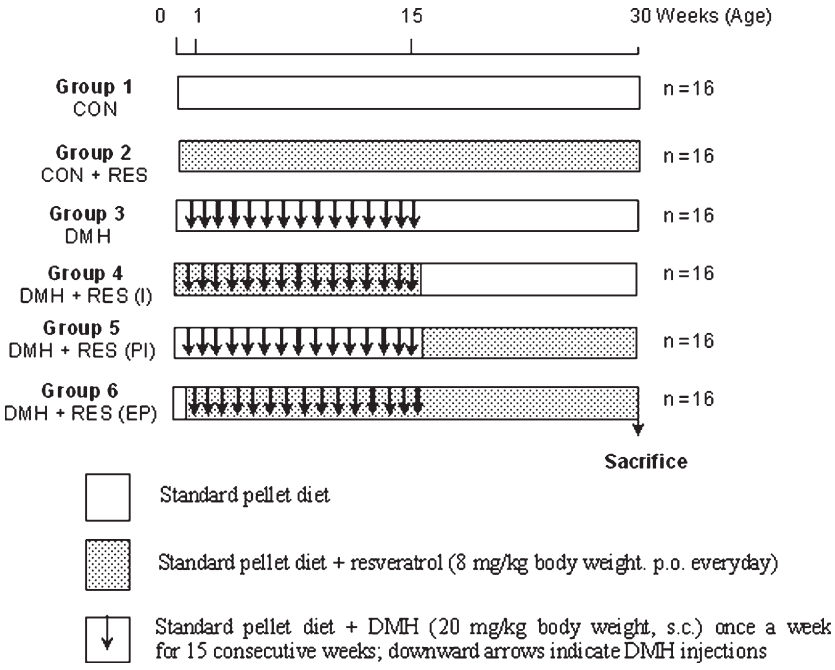

Fig. 1. Experimental design.

color developed was read at 560 nm colorimetrically by the method of Jiang et al. [17]. The levels of lipid hydroperoxides were expressed as mmol/mg tissue.

Levels of thiobarbituric acid reactive substances (TBARS) in tissue homogenates were estimated by the method of Okhawa et al. [18]. To 0.2 ml tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA were added. The mixture was made up to 4.0 ml with distilled water and then heated in a water bath at 95 °C for 60 min. After cooling, 1.0 ml of water and 5.0 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 600×g for 10 min, the absorbance of the organic layer was measured at 532 nm. 1,1,3,3-tetramethoxypropane was used as the standard. The levels of TBARS were expressed as mmol/mg tissue.

2.7. Determination of SOD and CAT enzyme activities

SOD was assayed according to the method of Kakkar et al. [19] and is based on the reduction of NBT^{2+} to a blue formazan (MF^{+}) by $O_2^{\bullet -}$. The color developed was measured spectrophotometrically at 550 nm and was expressed in terms of 50% inhibition of NBT reduction/min/mg protein.

CAT was assayed as described by Sinha [20]. The reaction mixture contained 1.0 ml phosphate buffer (0.01 M, pH 7.0), 0.1 ml tissue homogenate and 0.4 ml H_2O_2 (0.2 M). The reaction was stopped by the addition of 2.0 ml dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid, 1:3 ratio). The absorbance was measured calorimetrically at 620 nm and the values were expressed as μmol of H_2O_2 utilized/min/mg protein.

2.8. Determination of glutathione and glutathione dependent enzymes

The levels of tissue GSH (reduced) was determined by the method of Boyne and Ellman [21]. Tissue homogenates were immediately precipitated with 0.1 ml of 25% trichloroacetic acid and the precipitate was removed after centrifugation. Free SH groups were assayed in a total volume of 3.0 ml by the addition of 2.0 ml DTNB (0.6 mM), 0.9 ml phosphate buffer (0.2 M, pH 8.0) and 0.1 ml homogenate. The absorbance was recorded at 412 nm. GSH values were expressed as mmol of GSH/mg tissue.

GPX activity was measured by the method of Flohe and Gunzler [22]. Briefly, the reaction mixture contained 0.2 ml phosphate buffer (0.4 M, pH 7.0), 0.1 ml sodium azide (10 mM), 0.2 ml tissue homogenate, 0.2 ml GSH (30 mM) and 0.1 ml H_2O_2 (0.2 mM). The contents were incubated at 37 °C for 10 min, and the reaction was arrested by addition of 0.4 ml of 10% TCA and centrifuged. Supernatant was assayed for GSH content by using Ellman's reagent and the levels were expressed as μmol of GSH utilized/min/mg protein.

GST activity was determined spectrophotometrically by the method of Habig and Jakoby [23]. The reaction mixture contained 1.7 ml phosphate buffer (100 mM, pH 6.5), 0.1 ml GSH (30 mM) and 0.1 ml of CDNB (30 mM). After preincubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of 0.1 ml tissue homogenate and the absorbance was followed for 5 min at 340 nm. The specific activity of GST was expressed as μmol of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6×10^4 M/cm.

GSSG reductase activity was assayed spectrophotometrically by the method of Carlberg and Mannervik [24]. The amount of NADPH consumed during GSSG reduction was used as the index of enzyme activity. The assay mixture contained potassium phosphate buffer (50 mM, pH 6.6), GSSG (0.8 mM), NADPH (0.48 mM) and tissue homogenate in a total volume of 0.25 ml. The absorbance was recorded every 10 min for 1 h, relative to a control in which GSSG was omitted. The enzyme activity was expressed as μmol of NADPH oxidized/min/mg protein.

2.9. Estimation of non-enzymic antioxidants

Vitamin C was determined by the method of Omaye et al. [25]. To 0.5 ml tissue homogenate, 0.5 ml water and 1.0 ml of 5% TCA were added, mixed thoroughly and centrifuged. 1.0 ml supernatant was treated with 0.2 ml DTC reagent and incubated at 37 °C for 3 h. Then, 1.5 ml of 65% sulphuric acid was added mixed well and the solution was allowed to stand at room temperature for another 30 min and the color developed was read at 520 nm. The vitamin C content was expressed as mg/mg tissue.

Vitamin E was estimated by the method of Desai [26]. 1.0 ml tissue homogenate and 1.0 ml ethanol were thoroughly mixed, followed by the addition of 3.0 ml of petroleum ether, shaken rapidly and centrifuged. 2.0 ml supernatant was evaporated to dryness and 0.2 ml of 0.2% bathophenanthoraline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride (0.001 M) was added followed by 0.2 ml of *O*-phosphoric acid (0.001 M). The total volume was made up to 3.0 ml with ethanol and the color developed was read at 530 nm. The vitamin E content was expressed as mg/mg tissue.

Protein content was determined by the method of Lowry et al. [27] using bovine serum albumin as standard at 660 nm.

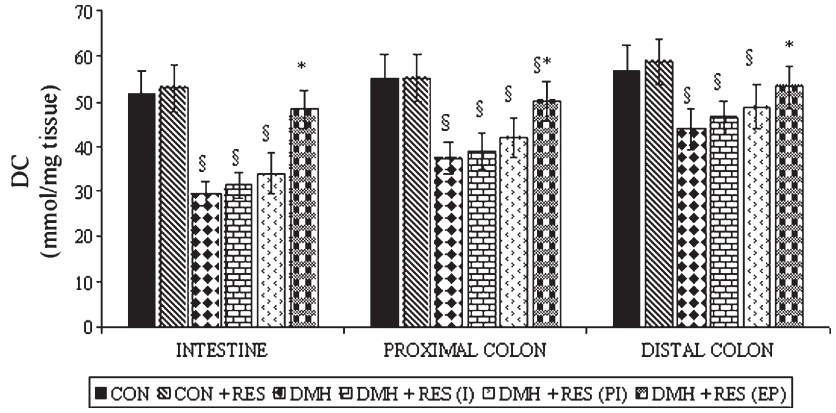

Fig. 2. Effect of resveratrol on tissue diene conjugates (DC) of control and experimental groups. The column heights are the means±S.D. for 8 values. Standard deviations are shown as bars. Statistically significant differences between control (CON) and other treatment groups are indicated by §($P<0.05$) and between DMH-treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)] by *($P<0.01$).

Data were analyzed by one-way analysis of variance (ANOVA) and a significant difference among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at $P<0.05$. All statistical analysis were made using SPSS 11.0 software package (SPSS, Tokyo, Japan).

3. Results

3.1. Effect of resveratrol on lipid peroxidation products

Figs. 2, 3 and 4 shows the levels of diene conjugates (DC), lipid hydroperoxides (LOOHs) and thiobarbituric acid reactive substances (TBARS) in intestines, proximal and distal colonic tissues of control and experimental rats respectively. The levels of DC, LOOH and TBARS were significantly ($P<0.05$) lower in DMH-treated rats (group 3) as compared to that of untreated control rats (group 1). Resveratrol supplementation showed near normal levels of tissue DC, LOOH and TBARS. The effect was more pronounced ($P<0.01$) when resveratrol was supplemented throughout the experimental period (group 6).

3.2. Effect of resveratrol on SOD and CAT

The results (Fig. 5 and 6) show a significant reduction ($P<0.05$) of SOD and CAT activities in DMH alone-treated rats (group 3) as compared to the control. But on resveratrol supplementation (groups 4–6) the SOD and CAT activities were significantly elevated ($P<0.05$) as compared to the unsupplemented DMH-treated group. The effect was more pronounced ($P<0.01$) when resveratrol was supplemented throughout the entire period (group 6).

3.3. Effect of resveratrol on glutathione and glutathione dependent enzymes

The levels of GSH and GR activity were significantly lowered in animals that were treated with DMH (group 3) as compared to control rats (Table 1). The levels/activities were significantly augmented on supplementation with resveratrol ($P<0.05$), whereas moderate but significant increase in the

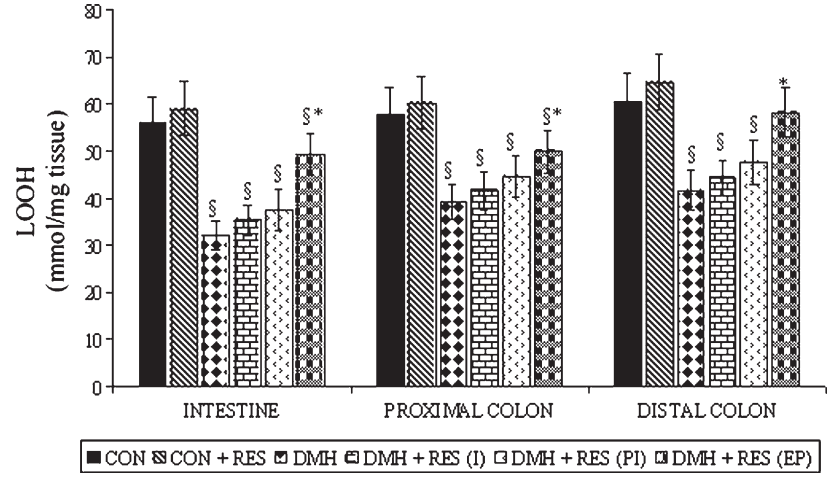

Fig. 3. Effect of resveratrol on tissue lipid hydroperoxides (LOOHs) of control and experimental groups. The column heights are the means±S.D. for 8 values. Standard deviations are shown as bars. Statistically significant differences between control (CON) and other treatment groups are indicated by §($P<0.05$) and between DMH-treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)] by *($P<0.01$).

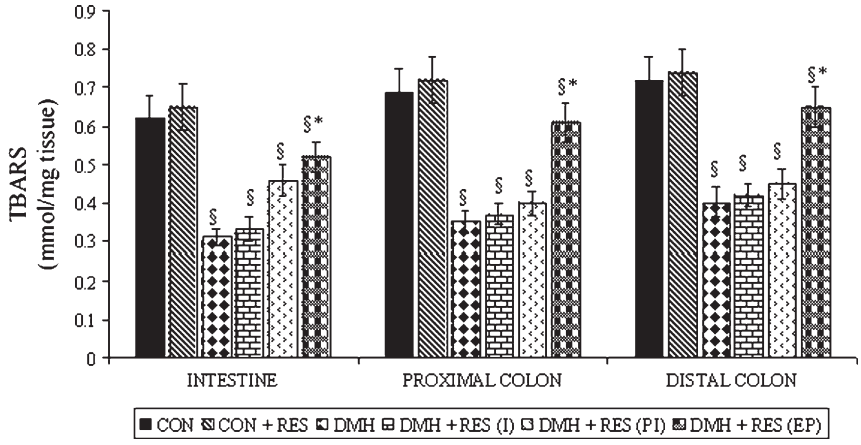

Fig. 4. Effect of resveratrol on tissue thiobarbituric acid reactive substance (TBARS) of control and experimental groups. The column heights are the means±S.D. for 8 values. Standard deviations are shown as bars. Statistically significant differences between control (CON) and other treatment groups are indicated by §($P<0.05$) and between DMH-treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)] by *($P<0.01$).

levels/activities were observed in the post-initiation resveratrol supplementation (group 5, $P<0.05$). But highly significant increase in the levels/activities ($P<0.01$) was observed only in the entire period resveratrol treatment (group 6).

The administration of DMH (group 3) in rats elevated the intestinal and colonic GPX and GST enzyme activities as compared to control. But on resveratrol supplementation (groups 4–6) the GPX and GST activities were significantly decreased ($P<0.05$) as compared to the unsupplemented DMH-treated group. The effect was more pronounced ($P<0.01$) when resveratrol was supplemented throughout the entire period (group 6).

3.4. Effect of resveratrol on ascorbate and α-tocopherol concentrations

The concentrations of tissue ascorbate and α-tocopherol were higher ($P<0.05$) on DMH treatment (group 3) as compared to control (Fig. 7 and 8). But on resveratrol supplementation (groups 4–6), the concentration of ascorbate and α-tocopherol was significantly decreased ($P<0.05$) as compared to the unsupplemented DMH-treated group. The effect was more pronounced ($P<0.01$) when resveratrol was supplemented throughout the entire period (group 6).

3.5. Effect of resveratrol on ACF formation

Rats in groups 1 and 2, that were not treated with DMH, showed no evidence of ACF formation in the colon. In group 3 rats, DMH treatment induced ACF (100.3±10.2) and the mean number of foci with more than four crypts (22.3±1.2) were observed (Table 2). Resveratrol supplementation (groups 4–6) significantly reduced the total number of ACF (50.4±5.3, 39.4±5.4 and 28.5±5.1) as well as the mean number of foci with more than four crypts (8.2±1.0, 7.7±1.0 and 6.2±1.2) as compared to DMH alone-treated rats (group 3).

The crypt height was (35.75±3.33) higher ($P<0.01$) in the colon of rats treated with DMH than in control. The crypt height in the resveratrol supplemented rats (groups 4–6) were 34.69±3.39, 33.84±3.30 and 27.25±2.49 respectively. The crypt height was significantly lower ($P<0.01$) in the entire

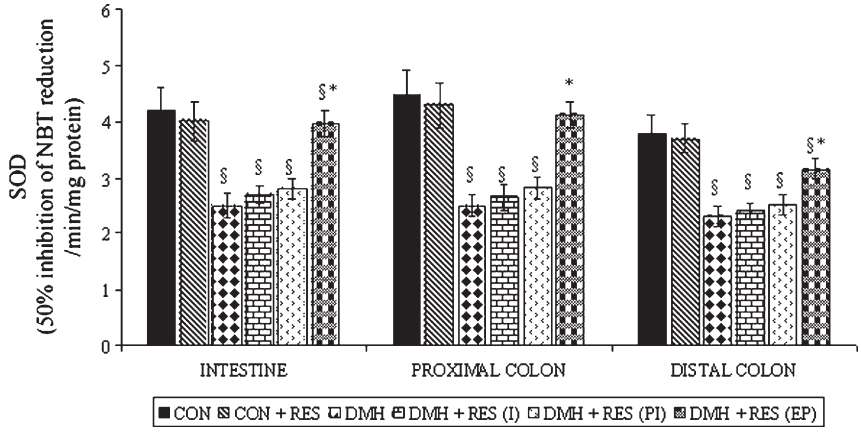

Fig. 5. Effect of resveratrol on tissue superoxide dismutase (SOD) activities of control and experimental groups. The column heights are the means ± S.D. for 8 values. Standard deviations are shown as bars. Statistically significant differences between control (CON) and other treatment groups are indicated by §($P<0.05$) and between DMH-treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)] by *($P<0.01$).

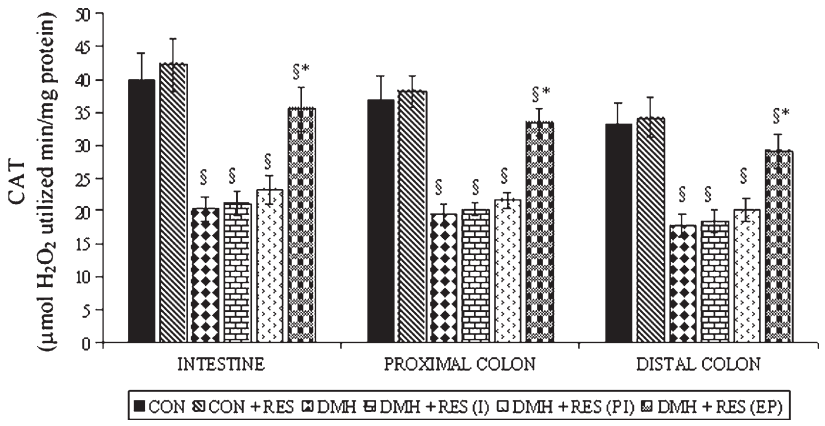

Fig. 6. Effect of resveratrol on tissue catalase (CAT) activities of control and experimental groups. The column heights are the means±S.D. for 8 values. Standard deviations are shown as bars. Statistically significant differences between control (CON) and other treatment groups are indicated by §($P<0.05$) and between DMH-treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)] by *($P<0.01$).

period resveratrol supplemented group as compared to DMH alone-treated rats.

3.6. Effect of resveratrol on tumor occurrence

Analysis of tumors according to the anatomical site and histological type revealed that most of the tumors in the proximal and distal colon were adenocarcinomas. Among the resveratrol supplemented groups, a marked decrease ($P<0.01$) in the adenoma/adenocarcinoma and their distribution along the proximal and distal regions of the colon (Table 3) was observed in entire period resveratrol supplemented animals (group 6) as compared to the other two dietary regimens (initiation; group 4 and post-initiation; group 5).

4. Discussion

Cancer can be inhibited at different stages of its development. Induction of antioxidants and detoxifying enzymes by anti-carcinogens appear to be a form of adaptation to metabolic stress [28]. Our results described here clearly indicate that resveratrol supplementation during the entire experimental period modulates lipid peroxidation and antioxidant defense with concomitant inhibition of ACF, adenoma/adenocarcinoma development in animals induced with colon carcinogen DMH.

Increased flux of oxy-radicals and loss of cellular redox homeostasis can cause oxidative stress and lead to tumorigenesis [29]. The colon-specific carcinogen DMH is metabolized to a methyl-free radical which in turn generates hydroxyl radical or

Table 1
Effect of resveratrol on glutathione and glutathione dependent enzyme activities in intestine, proximal and distal colon of control and experimental groups[a]

	CON	CON+RES	DMH	DMH+RES (I)	DMH+RES (PI)	DMH+RES (EP)
GSH (mmol/mg tissue)						
Intestine	21.14±2.05	22.10±2.08	13.94±1.32[b]	14.67±1.32[b]	15.93±1.37[b]	17.51±1.64[b,*]
Proximal colon	20.18±1.96	21.11±1.28	12.19±1.08[b]	13.08±0.75[b]	13.62±1.12[b]	17.02±0.98[b,*]
Distal colon	18.52±1.72	19.50±1.72	10.27±1.01[b]	11.15±0.99[b]	12.18±1.17[b]	16.22±1.44[b,*]
GST (μmol CDNB-GSH conjugate formed/min/mg protein)						
Intestine	1.80±0.17	1.72±0.17	3.20±0.27[b]	2.96±0.28[b]	1.91±0.18[b]	1.86±0.16*
Proximal colon	1.50±0.13	1.42±0.08	3.10±0.30[b]	2.87±0.25[b]	2.25±0.77[b]	1.61±0.09*
Distal colon	1.46±0.13	1.38±0.11	2.76±0.59[b]	2.85±0.24[b]	2.49±0.21[b]	1.60±0.26[b,*]
GPX (μmol GSH utilized/min/mg protein)						
Intestine	6.08±0.63	6.32±0.46	8.50±0.73[b]	8.09±0.79[b]	7.84±0.67[b]	7.16±0.64*
Proximal colon	5.81±0.51	5.53±0.33	7.90±0.77[b]	7.63±0.44[b]	6.89±0.61[b]	6.12±0.35*
Distal colon	5.10±0.50	5.01±0.44	6.92±0.64[b]	6.26±0.55[b]	5.97±0.53[b]	5.43±0.48*
GR (μmol NADPH oxidized/min/mg protein)						
Intestine	10.25±1.01	10.50±0.91	5.59±0.51[b]	5.93±0.34[b]	6.34±0.42[b]	9.50±0.55[b,*]
Proximal colon	11.50±1.12	11.65±1.10	6.50±0.56[b]	6.73±0.58[b]	7.42±0.53[b]	10.55±0.61[b,*]
Distal colon	12.10±1.05	12.50±0.86	7.20±0.61[b]	7.51±0.52[b]	8.07±0.60[b]	11.55±0.67*

[a] Results are means±S.D. for $n=8$.
[b] Different from control (CON); $P<0.05$.
* Statistically significant difference between unsupplemented DMH treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)]; $P<0.01$.

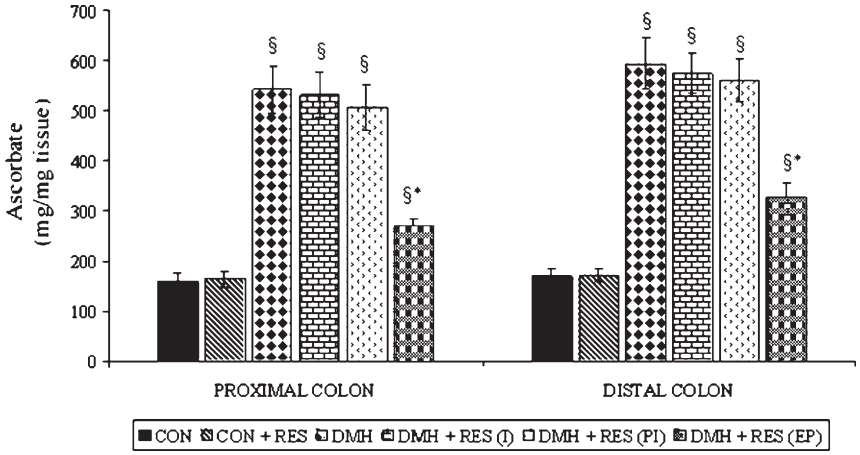

Fig. 7. Effect of resveratrol on tissue ascorbate levels of control and experimental groups. The column heights are the means ± S.D. for 8 values. Standard deviations are shown as bars. Statistically significant differences between control (CON) and other treatment groups are indicated by $^{\S}(P<0.05)$ and between DMH-treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)] by *($P<0.01$).

hydrogen peroxide in the presence of metal ions that may contribute to the initiation of lipid peroxidation. Our data are also in line with the previous studies which proved that rats administered DMH for longer periods have decreased tissue lipid peroxidation (DC, LOOH and TBARS) as compared to those of the control rat tissue [7]. An inverse relationship between the concentration of lipid peroxides and the rate of cell proliferation [30] and differentiation [9] is well documented. Moreover, a number of studies have demonstrated that tumor cells have reduced levels of phospholipids and polyunsaturated fatty acids (PUFA) [7]. The low content of PUFA in tumor cells can be attributed to the loss or decreased activity of δ-6- and δ-6-5-desaturases [30], lending support to the concept that the rate of lipid peroxidation is generally low in tumor cells.

The enzymes SOD, CAT and the glutathione system play a key role in the cellular defense against free radical damage [31]. Wide body of data indicates that animal tumor cells lack complex enzyme systems, which normally exert protection by scavenging toxic oxygen species such as superoxide radical, hydrogen peroxide and lipid hydroperoxides [32]. Our results show a significant decrease in the activities of SOD, CAT and GR ($P<0.01$) while the activities of glutathione utilizing enzymes such as GPX and GST were significantly increased ($P<0.01$) (almost doubled) in carcinogen-treated rats and serve as markers for (pre) neoplastic tissues [33]. Colonic mucosal GSH (ubiquitous cellular reductant) levels were lowered in DMH-treated rats. This suggests that this tripeptide may be involved in the detoxification and possible repair mechanisms in the colonic mucosa [34].

SOD, CAT, GSH and GR replenishment (increase) on resveratrol supplementation for the entire period reflects a favorable balance between potentially harmful oxidants and protective antioxidants. Furthermore, elevated SOD and CAT activities can play an inhibitory role on cell transformation [35]. CAT has been found to significantly decrease chromosomal aberrations, and also to delay or prevent the onset of spontaneous neoplastic transformation in mouse fibroblasts and epidermal keratinocytes [35]. Many findings report that decreased CAT activity in tumor cells is somehow compensated by an increase in GPX activity, which in turn prevents tumor cells from

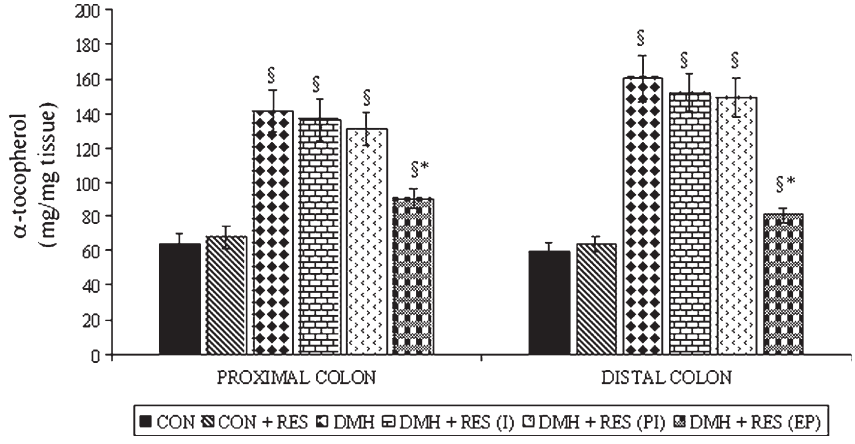

Fig. 8. Effect of resveratrol on tissue α-tocopherol levels of control and experimental groups. The column heights are the means ± S.D. for 8 values. Standard deviations are shown as bars. Statistically significant differences between control (CON) and other treatment groups are indicated by $^{\S}(P<0.05)$ and between DMH-treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)] by *($P<0.01$).

Table 2
Effect of resveratrol on multiplicity, size, total number of aberrant crypt foci (ACF) containing ≥4 aberrant crypt (AC) and crypt height of control and experimental groups[a]

Groups	Total no. of ACF	Total no. of ACF containing ≥ 4 AC	Multiplicity (no./rat)	Size (ACF/AC)	Crypt height
CON	–	–	–	–	23.08±1.85
CON+RES	–	–	–	–	24.25±2.16
DMH	100.3±10.2	22.3±1.2	18.05±1.40	0.55±0.04	35.75±3.33
DMH+RES (I)	50.4±5.3	8.2±1.0	8.56±0.76	0.58±0.05	34.69±3.39
DMH+RES (PI)	39.4±5.4	7.7±1.0	6.30±2.09	0.62±0.20	33.84±3.30
DMH+RES (EP)	28.5±5.1*	6.2±1.2*	3.70±1.43*	0.77±0.30*	27.25±2.49*

[a] Results are means±SD for $n=8$.
* Statistically significant difference between unsupplemented DMH treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)]; $P<0.01$.

peroxidative attack. The overexpression of GST enhances the production of eicosonoids, another common attribute observed in many tumors [32]. Furthermore, the ratio among these antioxidant enzymes is important, as any imbalance will result in the accumulation of toxic-free radicals that cause cell damage [33]. Previous findings [36] firmly establish GST inhibition as one of the major mechanism to explain the chemopreventive efficacy of phytochemicals. Reduction in GPX and GST activities on resveratrol supplementation shows that resveratrol may play a role in maintaining the balance between these antioxidant enzymes, which is in harmony with the previous reports [36]. However, the changes in GPX and GST enzyme activities might be due to the malignant state, and recovery of the enzyme activities could help to reverse malignancy.

Our results have demonstrated elevated levels of the water soluble antioxidant ascorbate (vitamin C) and lipid soluble antioxidant α-tocopherol (vitamin E) in neoplastic tissues as compared to normal tissues which correlate with the previous studies [37, 38]. Cancer cells readily take up vitamin C in the oxidized form through GLUTs [38], but the precise function of vitamin C in neoplastic tissues is unknown. The high vitamin E levels in tumor cells can be due to the decreased content of PUFA, which might contribute to the low rate of lipid peroxidation [30]. On resveratrol supplementation, the levels of vitamins C and E were lower, which may be correlated with the decreased uptake of ascorbate by malignant cells or to the decreased resistance of transformed cells to lipid peroxidation.

Present findings show that resveratrol suppress ACF development, adenoma/adenocarcinoma occurrence which might be a useful outcome for evaluating chemopreventive potential in colon carcinogenesis. Larger ACF (four or more aberrant crypts per focus) are considered more likely to progress into tumors [15]. Resveratrol supplementation suppressed not only the total number of ACF, but also affected the development of multi crypt foci containing 4 or more AC/foci in the colon. Hyperplasia was assessed by counting the number of cells per crypt column (crypt height). The number of cells in the crypt column was decreased on resveratrol supplementation. In addition, the decrease in crypt height might be correlated with a significant decrease in hyperplastic lesions [39].

The dose 8 mg/kg body weight resveratrol used in this experiment was selected on the basis of our preliminary dose fixation study (data not shown) and from many other earlier reports. It has been demonstrated that the antioxidant property of resveratrol in different experimental models with various doses ranging from 8 to 40 mg/kg body weight [40]. Moreover, Jang et al. proved that resveratrol 8 mg/kg body weight acts as an effective chemopreventive agent against mouse skin cancer model by blocking the cancer development and formation at various stages including initiation, promotion and progression [14]. Human equivalent dose (HED) is a dose in humans anticipated to provide the same degree of effect as that observed in animals at a given dose. 8 mg/kg body weight resveratrol in rats equals to 1.29 mg/kg body weight in humans. This dose cannot be directly correlated with the daily dietary/wine intake by the human beings. Rather resveratrol can be used as a daily supplement to human beings.

Polyphenols and flavanoids are known to prevent oxidative damage by its ability to scavenge reactive oxygen species such as hydroxyl radical and superoxide anion. The cytotoxic action of resveratrol against cancer cells may be through mobilization of endogenous copper and the consequent prooxidant action, which might be one of the mechanisms involved in ROS-

Table 3
Effect of resveratrol on the distribution of colonic adenoma and adenocarcinomas in DMH-treated rats[a]

Groups	Entire large bowel			Proximal colon			Distal colon		
	Total	Adenoma	Adenocarcinoma	Total	Adenoma	Adenocarcinoma	Total	Adenoma	Adenocarcinoma
DMH	19	7	12	7	2	5	12	5	7
DMH+RES (I)	12	5	7	4	1	3	8	4	4
DMH+RES (PI)	9	5	4	3	2	1	6	3	3
DMH+RES (EP)	4*	4*	0*	0*	0*	0*	4*	4*	0*

[a] Results are means±S.D. for $n=8$.
* Statistically significant difference between unsupplemented DMH treated group (DMH) and resveratrol supplemented groups [DMH+RES (I), DMH+RES (PI), DMH+RES (EP)]; $P<0.01$.

mediated tumor cell apoptosis and cancer chemoprevention [41]. Thus, the significant increase in lipid peroxidation products observed on chronic resveratrol supplementation (group 6) may be correlated with its prooxidant property.

Overall, we speculate that chronic administration of resveratrol (entire period) to carcinogen-exposed rats ameliorate the deleterious effects of DMH-mediated oxidative stress, ACF development, adenoma/adenocarcinoma occurrence which may be attributed to its antioxidant and antiproliferative properties.

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