

MODULATORY INFLUENCE OF DIETARY GALLIC ACID ON MOLECULAR MARKERS IN 1, 2 DIMETHYLHYDRAZINE–INDUCED COLON CARCINOGENESIS IN AN EXPERIMENTAL RAT MODEL

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ABSTRACT

Our aim was to explore the modulatory effect of gallic acid (GA) preneoplastic and neoplastic markers employing 1,2-dimethylhydrazine (DMH) induced colon cancer in male Wistar rats as an experimental model during the different stages of carcinogenesis. Group 1 served as control, group 2 rats were given GA, 50mg/kg body weight by intragastric intubation everyday; groups 3-6 were injected DMH (s.c. injections, 20mg/kg body weight) once a week for the first 15 weeks. Group 4 rats received GA as in group 2 starting 1 week before DMH injections and continued till the final exposure [DMH + GA (I)]. Group 5 rats received GA as in group 2 after the cessation of DMH injections and continued till the end [DMH + GA (PI)] of the experimental period. The rats in group 6 were supplemented with GA as in group 2 from the day of carcinogen treatment and continued till the end of the entire experimental period of 32 weeks [DMH+GA (EP)]. Colon tissues were analyzed for the expression of β -catenin, proliferating cell nuclear antigen (PCNA) and argyrophilic nucleolar organizer regions (AgNORs) by using immunohistochemistry and silver staining. Immunoblotting was employed to study cyclin D1 expression and preneoplastic changes. GA inhibited DMH-induced colon cancer by decreasing tumour incidence, multiplicity and also the formation ACF. Supplementation with GA to DMH-treated rats decreased the levels of β -catenin, PCNA, AgNORs and cyclin D1. Since colon cancer is highly sensitive to dietary intervention, adults who may have preneoplastic lesions in their colon may be benefited by GA.

KEY WORDS

Chemoprevention; Colon cancer; Gallic acid; Cell proliferation; Preneoplastic Lesions

INTRODUCTION

Colorectal cancer is ranked the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the Western world¹. Colon cancer arises due to diverse genetic and epigenetic changes in the colonic epithelium. In addition to genetic predisposition, diet is an important risk factor for colon cancer. Accumulating evidence suggests an association between high fat intake and an increased risk of colorectal cancer². 1, 2-

dimethylhydrazine (DMH) is a colon specific carcinogen and an alkylating agent. DMH is believed to form active intermediates including azoxymethane and methyloxymethanol in the liver, which are subsequently transported into the colon through bile. Methylazoxymethanol is decomposed to form methyldiazonium ion, which methylates cellular components and in turn produces tumours in the colon³.

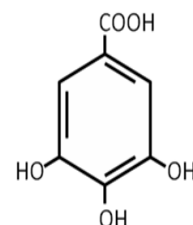
Aberrant crypt foci (ACF), a colon carcinoma precursor in humans and rats, are a feasible tool to distinguish between the polyp types and can serve as a sensitive, realistic and rapidly appearing biomarker supported by the presence of histopathological intraepithelial neoplasia⁴. ACF are readily discernible pre-adenomatous morphologically putative lesions within the colonic mucosa of rodents and cancer patients, and they may contribute to the stepwise progression of colon cancer⁵. ACF appear predominantly in the distal colon early in carcinogenesis; subsequently, they appear in the proximal colon and may exhibit focal expansion⁶. ACF grow via a mechanism of crypt formation, and ACF with more crypts indicate a more advanced growth stage⁷. DMH induced ACF are histologically categorized into two types; namely, nondysplastic and dysplastic ACF (DACF), the latter harboring β -catenin and/or Apc mutations, and demonstrating a mutation spectrum very similar to that observed in colon cancers. In carcinogen treated rodents with sporadic colorectal cancer, the number of tumors is minuscule compared with the large number of ACF, indicating that only a very small fraction of ACF progresses to the stage of a tumor. This is consistent with the observation that a large fraction of ACF is hyperplastic whereas only a small fraction of ACF shows dysplasia, a hallmark of malignant potential⁸. It has been proposed that only the dysplastic ACF progress to adenomas and adenocarcinomas and these lesions are closely related to Apc mutations.

Other types of preneoplastic lesions may also exist and several candidates have been proposed as more relevant surrogate endpoints than ACF. These include β -catenin accumulated crypts, mucin-depleted foci, microadenomatous lesions in the ApcMin mouse, and dysplastic ACF (DACF)⁹.

The histopathological technique of one-step silver staining of argyrophilic proteins associated with nucleolar organizer regions (AgNORs) are specifically related to loops of DNA containing the sites of ribosomal RNA and play a role in the synthesis of ribosomes¹⁰. The number and size of ribosomes and of AgNORs are related to the proliferation, differentiation and malignant transformation of cells^{11,12}. In spite of the potential value of this method in oncology, there are many instances in which differences between the AgNORs of benign and malignant tissues are not

significant¹³. In colonic cancer the AgNOR scores may be important not only in the diagnosis of a potentially malignant lesion but also in defining the margins of abnormal tissue.

Proliferating cell nuclear antigen (PCNA) is a 36 KDa nuclear protein which function as an auxiliary protein for DNA polymerase δ and is an absolute requirement for DNA synthesis^{14,15}. It is a stable cell-cycle regulated nuclear protein that is expressed differentially during the cell cycle and whose rate of synthesis is correlated directly with the proliferative rate of cells. The levels of PCNA increase in nucleus during the late G1-phase, immediately before the onset of DNA synthesis, become maximal during the S-phase; decline during the cell cycle, two populations of cells with differing PCNA can be distinguished. Abnormal cellular proliferation is one of the crucial mechanisms in carcinogenesis¹⁶.



**Fig 1: Structure of Gallic acid
(3, 4, 5- trihydroxy benzoic acid)**

Epidemiological data available strongly correlates the dietary intake of food, vegetables and medicinal plants and reduced risk of cancer in experimental animals and humans. In this regard, naturally occurring biologically active polyphenols derived from common dietary sources are gaining interest as potential cancer therapeutics¹⁷. Phenolic compounds have been reported to have a capacity to scavenge free radicals. Gallic acid [3,4,5- trihydroxy benzoic acid, GA **Fig. 1**] is a polyhydroxy phenolic compound, which can be found in various natural products, like gallnuts, tea leaves, bark, green tea, apple-peels, grapes, strawberries, pineapple, banana lemons, and in red and white wine¹⁸. GA is a strong natural antioxidant¹⁹ and is pharmacologically active as an antiallergic, antimutagenic, anti inflammatory and anticarcinogenic agent²⁰. It inhibits melanogenesis which may be related to GA's antioxidant activity in scavenging reactive oxygen species.

The purpose of the present study was to investigate the modulating effect of GA administered during the initiation, post-initiation and also throughout the entire period of the study on the development of ACF, dysplastic ACF (DACF), the incidence of tumors, and the expression of cell proliferation markers.

MATERIALS AND METHODS

(i) Animals

The experiments were carried out using male Albino Wistar rats, (160-180g) obtained from the Central Animal House, Annamalai University, India. The rats were kept in polypropylene cages (4 per cage) with paddy husk for bedding and fed modified pellet diet (**Table 1**) during the initial period of one week for

acclimatization. Thereafter, the rats were randomly divided into six groups, maintained under controlled conditions of temperature ($24 \pm 2^\circ\text{C}$) humidity ($50 \pm 10\%$) and a 12 h light/dark cycle. Commercial pellet diet containing 4.2% fat (Hindustan Lever Ltd, Mumbai, India) was powdered and mixed with 15.8% peanut oil, making a total of 20% fat in the modified pellet diet. Rats were allowed free access to this modified powdered pellet diet and tap water throughout the experimental period of 32 weeks. The rats were cared for in compliance with the principles and guidelines of the Ethical Committee for Animal Care of Annamalai University in accordance with the Indian National Law on Animal Care and Use (Reg. No.160/1999/CPCSEA/842).

Table 1: Composition of the modified pellet diet

Ingredients	Commercial pellet diet 84.2%	Peanut oil 15.8%	Total 100%
Proteins	17.7	-	17.7
Fat	4.2	15.8	20.0
Carbohydrates	50.5	-	50.5
Fiber	3.4	-	3.4
Minerals	6.7	-	6.7
Vitamins	1.7	-	1.7

Table 2: Body weight changes on treatment with GA and DMH

Groups	Initial body weight (g)	Final body weight (g)
Control	163.7 $\pm$ 15.7	262.5 $\pm$ 25.2 ^a
Control + GA	158.5 $\pm$ 15.2	260.5 $\pm$ 25.0 ^a
DMH	163.4 $\pm$ 15.7	229.7 $\pm$ 22.1 ^b
DMH + GA (I)	158.7 $\pm$ 15.2	255.0 $\pm$ 24.5 ^c
DMH + GA (PI)	148.3 $\pm$ 14.2	250.4 $\pm$ 24.1 ^c
DMH + GA (EP)	153.0 $\pm$ 14.7	258.4 $\pm$ 24.8 ^d

Values are expressed as means $\pm$ S.D. of each group. Values not sharing a common superscript letter (a-d) differ significantly at $P < 0.05$ (analysis of variance followed by DMRT).

(ii) Chemicals

1,2-dimethylhydrazine (DMH) and gallic acid (GA), were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals and solvents used were of analytical grade and obtained from Hi-Media Laboratories, Mumbai.

(iii) Carcinogen administration

DMH was dissolved in 1mM EDTA, adjusted to pH 6.5 with 1mM NaOH and administered subcutaneously in the right thigh of rats at a dose of 20 mg/kg body weight once a week for the first 15 weeks.

(iv) Preparation of gallic acid

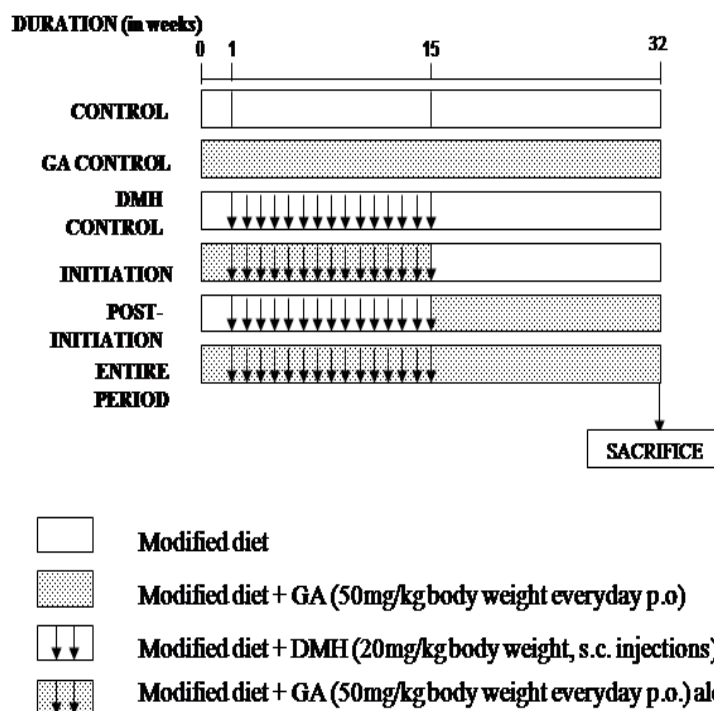
Gallic acid (GA) was solubilized in water just before treatment and was administered everyday orally at the dose of 50mg/kg body weight²¹ for 32 weeks.

(v) Treatment regimen

Group 1 served as control, group 2 rats were given GA, 50mg/kg body weight by intragastric intubation everyday; groups 3-6 were injected DMH (s.c. injections, 20mg/kg body weight) once a week for the first 15 weeks. Group 4 rats received GA as in group 2 starting 1 week before DMH injections and continued till the final exposure [DMH + GA (initiation-I)]. Group 5 rats received GA as in group 2 after the cessation of

DMH injections and continued till the end [DMH + GA (post-initiation - PI)] of the experimental period. The rats in group 6 were supplemented with GA as in group 2 from the day of carcinogen treatment and continued till the end of the entire experimental period of 32 weeks [DMH+GA (Entire period-EP)]. For more clarity experimental protocol is shown in the following figure.

Figure 2 Experimental protocol



(vi) Termination of the study

The experiment was terminated at the end of 32 weeks, food was withheld over night, the rats anesthetized (i.p. administration of ketamine hydrochloride, 30 mg/kg body weight), and sacrificed by cervical dislocation. Blood was collected in heparinized tubes and plasma was separated by centrifugation at 2000×g for 10 min. After the separation of plasma, the buffy coat was removed and packed cells (RBC) were washed thrice with cold physiological saline. Erythrocyte lysate was prepared by lysing a known volume of RBCs with hypotonic phosphate buffer, pH 7.4 and centrifuged at 3000×g for 10 min at 4°C and the hemolysate separated. The tissues (proximal colon, distal colon and liver) were immediately dissected out and rinsed with 0.9% NaCl and homogenized in the appropriate buffer using a tissue homogenizer.

(vii) Determination of aberrant crypt foci (ACF) and dysplastic ACF (DACF)

At the end of the 32-week study, rat colons were removed and flushed with potassium phosphate buffered saline (0.1M, pH 7.2). Colons were split open longitudinally and placed on strips of filter paper with their luminal surfaces open and exposed. Another strip of filter paper was placed on top of the luminal surface. The colons were then secured and fixed in a tray containing 10%buffered formalin overnight. Each of the fixed colon was cut into proximal and distal portions of equal lengths and each portion was further cut into 2cm long segments. Each segment was placed in a petridish and stained with 0.2% methylene blue solution for 2min. The segments were examined using a light microscope at low magnification to score the total number of ACF as well as the number of crypts per focus. ACF were distinguished from normal crypts

by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface of cells. ACF in the colon were counted as described by²². Subsequent decolourization with 70% methanol for 5 min revealed dysplastic ACF which retained blue staining and most of these showed dysplasia by histological analysis.

(viii) Silver staining

3µm thick colonic sections were sliced and embedded in paraffin wax. Staining for argyrophilic nucleolar organizer regions (AgNORs) was carried out by the method of²³. Silver colloid solution was made using 2% gelatin in 1% aqueous formic acid. This solution was mixed with twice its volume of 50% aqueous silver nitrate solution. Sections of the colon were exposed to the staining solution for 40 min at room temperature in the dark. The slides were washed in distilled water for 10 min, rehydrated through graded alcohol, cleared in xylene, and mounted using D.P.X. To determine the AgNORs count, the cell nuclei of 25 well-oriented crypts were used. AgNORs were visualized as distinct silver positive black dots. AgNORs were counted on silver stained sections under a microscope at a magnification of 40X.

(ix) Immunohistochemistry

4 µm thick paraffin-embedded sections were heated for 60 min at 60°C, deparaffinized in xylene, and rehydrated through graded alcohols at room temperature. Tris-HCl buffer (0.05 M; pH 7.6) was used to prepare solutions and for washes between various steps. Sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated with primary antibodies against the proteins PCNA and β-catenin (BioGenex Laboratories, Inc. San Ramon, CA, USA) for one hour. Horseradish peroxidase activity was visualized after treatment with H₂O₂ and diaminobenzidine (DAB) hydrochloride for 5min using the labeled streptavidin- biotin method (BioGenex Kit, San Ramon, USA). Immunoreactivities were regarded as positive if the apparent staining were detected in the cytoplasm and/or nuclei. The PCNA labeling index was determined by calculating the ratio of PCNA-positive nuclei/total number of nuclei.

(x) Immunoblotting of cyclin D1

Colonic proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes as described by²⁴. Briefly, the presoaked membranes were blocked in 5% non fat dry milk in Tris-buffered saline (TBS) for 2–4 h at room temperature and the immunodetection of colonic cyclin D1 was performed using primary antibody raised against cyclin D1 (rabbit polyclonal, Santa Cruz Biotechnology, UK). The membrane was washed thrice in TBS and incubated with secondary horseradish peroxidase-linked anti-rabbit IgG antibody (Santa Cruz Biotechnology, UK). Protein-antibody complexes were detected by the addition of (DAB) as a substrate. The blots were documented using Gel Documentation system (BioRad) and the intensity of the protein bands in the blot was quantified using the quantity one software.

Statistical analysis

All the biochemical analyses are expressed as means ± SD. Data were analyzed by one-way analysis of variance (ANOVA) and the 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).

RESULTS

1. Frequency of ACF and dysplastic ACF at the end of the study:

The incidence of ACF is shown in the long-term study (**Figure 3**). ACF developed in rats treated with DMH, with or without GA feeding (groups 3–6). No ACF were present in the control and control + GA rats (groups 1 and 2). The frequency of ACF/colon in the rats supplemented with GA during post-initiation and during the entire period of the experiment (groups 5 and 6, respectively) was significantly reduced than that of DMH alone treated rats (group 3) ($P < 0.05$). The effect was more pronounced in the rats supplemented with GA for the entire study period (group 6). Dysplastic ACF is shown in **Figure 4**.

Figure 3 Topographical view of ACF in the colonic mucosa of control and experimental rats

Fig. a and b: control and control + GA treated rat colons show normal crypts

Fig. c: DMH treated rat colon shows two aberrant crypt foci having more than 8 crypts

Fig. d: DMH+GA (I) treated rat colon shows five aberrant crypts

Fig. e: DMH+GA (PI) treated rat colon shows four crypt

Fig. f: DMH+GA (EP) treated rat colon shows three crypts

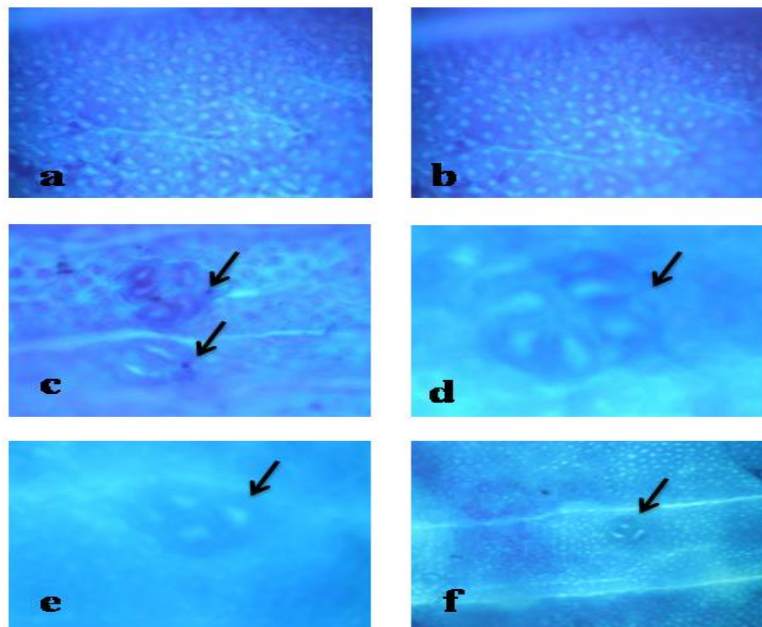


Figure 4: Topographical view of dysplastic ACF in the colonic mucosa of control and experimental rats stained with methanol, and decolourized

Fig. A: DMH treated rat colon shows dysplastic ACF with six crypts which resists methanol, decolourization after methylene blue staining

Fig. B: DMH+GA (I) shows dysplastic ACF with three crypts

Fig. C: DMH+GA (PI) shows dysplastic ACF with three crypts

Fig. D: DMH+GA (EP) shows dysplastic ACF with two crypts

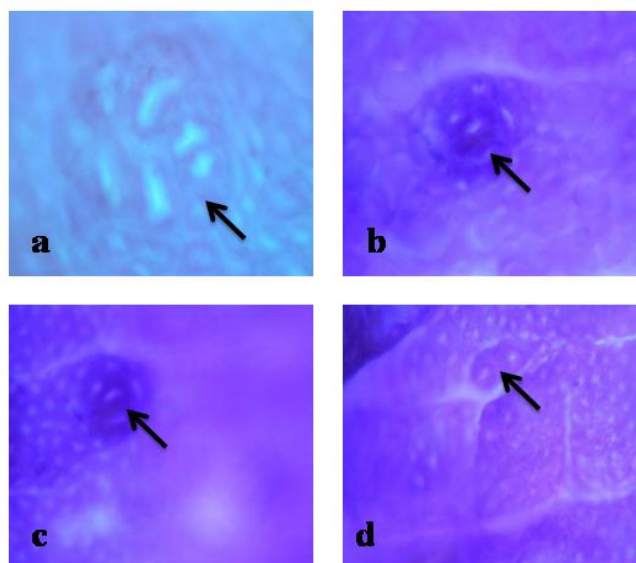


Figure 5 Immunohistochemical staining of colonic β -catenin accumulated crypts (40X)

Fig. A and B: Colon of control and GA control rats showing normal staining.

Fig. C: Colon of DMH alone treated rat shows β -catenin accumulation in the cytoplasm (dotted arrows).

Fig. D, E and F: Colons of GA supplemented DMH treated rats during the initiation, post-initiation stages of carcinogenesis and the entire study period exhibiting both moderate membrane staining and mild cytoplasmic staining.

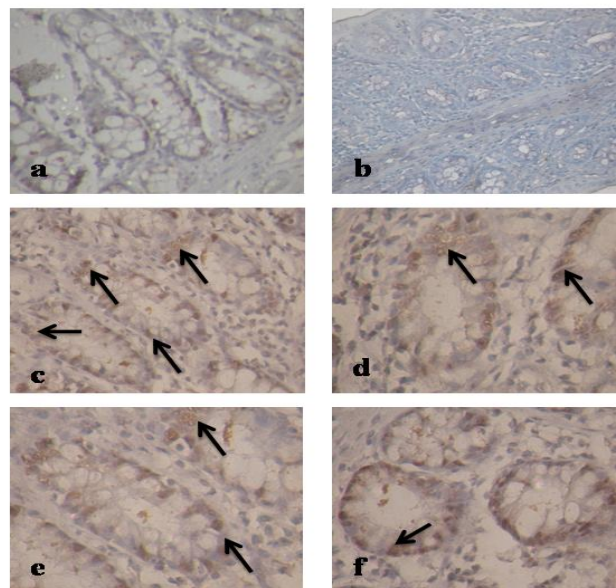


Figure 6 Immunohistochemical staining of colonic PCNA (40X)

Fig. A and B: Colon of control and GA control rats showing PCNA negative nuclei.

Fig. C: Adenocarcinoma of the colon with irregular glands exhibiting intense nuclei positivity showing PCNA labeling index of ~88%. Fig. D, E and F: PCNA expression in the colonic tissues of DMH-treated rats supplemented with GA during the initiation, post-initiation and the entire period treatment regimens showing scarce nuclear staining.

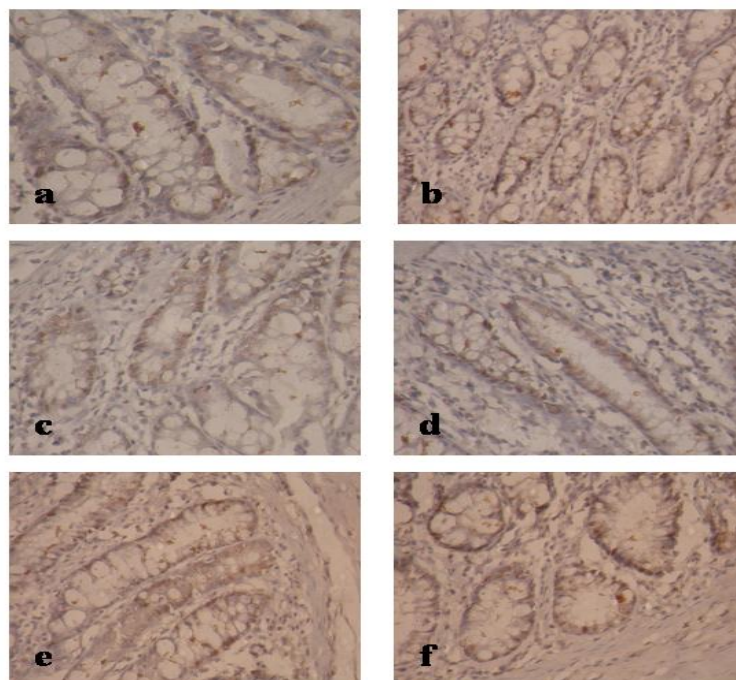


Figure 7 Silver staining pattern of AgNORs in the colonic tissue (40X)

Fig. A and B. Normal colonocytes lack silver positive black dots.

Fig. C: Adenocarcinoma of the colon with irregular glands exhibiting cluster of intense silver positivity (arrow pointed).

Fig. D, E and F: Silver staining was decreased significantly upon GA supplementation during the initiation, post-initiation and the entire period treatment regimens.

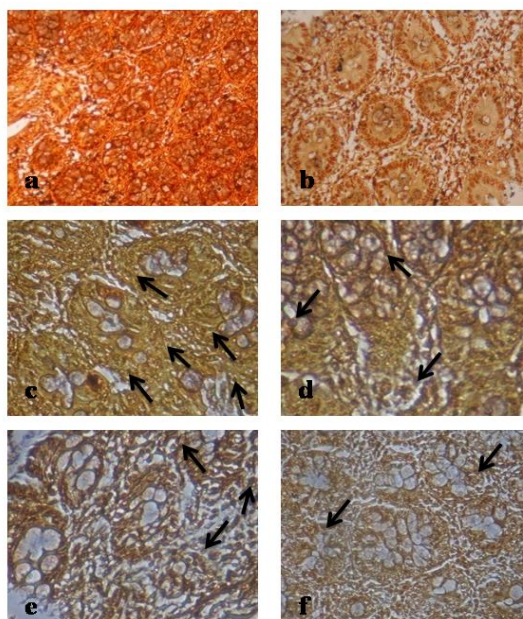
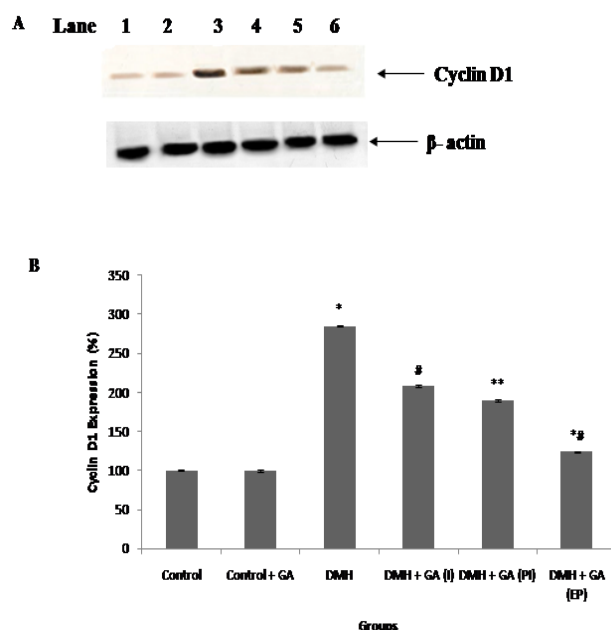


Figure 8 Expression of cyclin D1 in the colon of control and experimental rats.

Fig. A: Western blot analysis of colonic cyclin D1 expression of six experimental groups.

Fig. B: Each lane was analyzed by densitometry and the expression in control was considered as 100%. The column heights are the means $\pm$ S.E. of six determinants. Standard errors are shown as error bars. *significantly ($P < 0.05$) different from control groups, #significantly different from DMH alone treated groups, **significantly ($P < 0.05$) different from DMH+GA (PI) groups, *#Significantly ($P < 0.01$) different from DMH+GA (I) and DMH+GA (PI) groups). Groups with different superscripts differ significantly at $P < 0.05$. (DMRT).



2. Effect of GA on DMH-induced activation of colonic β -catenin:

β -Catenin accumulated crypts (BCAC) were analysed using immunostaining to assess the extent of preneoplastic lesions. **Figure 5** shows the representative photomicrographs of the immunohistochemical staining for BCAC. Control and gallic acid treated control rats showed intact membrane positivity (black arrow) for β -catenin whereas DMH alone treated rats showed intense cytoplasmic positivity indicating β -catenin accumulation in the cytoplasm as a consequence of tumourigenesis. GA supplementation during the initiation, post-initiation and entire period stages showed relatively lower levels of cytoplasmic β -catenin in the colon tissues as compared to rats treated with DMH alone. The inhibition of BCAC in DMH treated rats supplemented with GA throughout the study period (group 6) was more pronounced as compared to the other GA supplemented groups.

3. Effect of DMH and GA on colonic cell proliferation markers:

Figure 6 shows the immunohistochemical staining of PCNA in the colon of control and experimental rats. Rats treated with DMH alone showed intense nuclear staining for PCNA as compared to the control rats and GA supplemented control rats. GA supplementation to DMH treated rats during the initiation, post initiation stages and the entire study period showed decreased staining for PCNA. The effect of GA in reducing cell proliferation was more pronounced in the rats belonging to the post-initiation and entire period dietary regimens.

Figure 7 depicts the quantification and representative photomicrographs of AgNORs in the colonic crypts of control and experimental animals. The mean number of AgNORs / nucleus in carcinogen-alone exposed rats (group 3) was the highest (arrow pointed to circle engulfing the clusters of silver positive black dots) and that in DMH treated rats supplemented with GA throughout the study period (group 6) was the lowest in all the groups. The number of AgNORs / nucleus was significantly decreased in all the three GA supplemented DMH treated rats (group 4-6) as compared to the unsupplemented DMH-treated rats (group 3).

To further confirm the antiproliferative potential of GA, another proliferation marker, cyclin D1, was assayed in the colonic tissue extracts by immunoblotting (**Figure 8**). Cyclin D1 expression was significantly increased in DMH alone treated rats as compared to control rats. GA supplementation during the initiation, post-initiation stages and the entire study period significantly decreased cyclin D1 expression levels as compared to DMH alone-treated rats (group 3). There was no significant difference in PCNA labelling index, AgNORs and cyclin D1 levels between the control and GA supplemented control rats.

DISCUSSIONS

The results of the present study demonstrate that dietary supplementation with gallic acid (GA) during either the initiation, post-initiation or throughout the entire study period significantly inhibited DMH-induced colon carcinogenesis in rats. Our findings indicate that GA feeding at the dose of 50 mg/kg body weight is effective in the suppression of ACF development in the large bowel, which correlates with the tumour incidence²⁵. The purpose of this investigation, a part of a large-scale study is to prove the chemopreventive efficacy of the polyphenol, GA, using an established colon cancer model.

Several studies have suggested the growth features of ACF and dysplastic ACF and their location as a measure of the biological efficacy of the modifiers of colon carcinogenesis²⁶. ACF and dysplastic ACF may represent the earliest detectable lesions in the development of colon cancer. Considerable evidence support the concept that ACF are indeed preneoplastic lesions and that the number of ACF is predictive of eventual tumour formation²⁷. Various studies also suggest that dysplastic ACF/ACF are the precursors of colon cancer in humans and rodents²⁸. In our present study increased crypt size was observed in DMH-treated rats and moreover, at the end of the 32-week study the percentage of ACF in rats treated with DMH alone was 90% and each contained not less than four crypts/foci. In this context²⁹ have shown that increased number of ACF may reflect the initiation step of colorectal carcinogenesis, while the progressive increase in the number of crypts per ACF may correspond to the promotion step of colon

tumourigenesis. On the other hand, when GA was investigated for its chemopreventive efficacy against DMH-induced putative preneoplastic foci in the rat colon, it became clear that this colorant reduced the number of aberrant crypts/rat, but not the induction of ACF itself, when it was administered after carcinogen treatment. We also observed that the inhibitory effect of GA on the development of ACF and dysplastic ACF was more pronounced in the entire period treatment regimen (group 6) as compared to the other treatment groups. The above data are further supported by the crypt multiplicity findings. Moreover the activity of GA to reduce the number of ACF seems to be mainly in the promotion stage of colon carcinogenesis.

ACF with severe dysplasia has been identified as the actual precursor of colonic adenomas and adenocarcinomas. The presence of β -catenin accumulated aberrant crypts (BCAC)³⁰ in carcinogen treated rat colon made significant advances recently in the identification of events leading to tumour development. As described earlier, the morphological features of these lesions resemble those of advanced ACF³¹, strongly suggesting the plausibility that they are subtypes of advanced ACF. BCAC, which feature nuclear accumulation of the protein frequently have β -catenin gene mutations and has been demonstrated in the cross-section of colon mucosa. BCAC have premalignant characteristic resembling cancers and can be detected in all segments of the colon within five weeks of carcinogen treatment. It is also known that paneth-like cells are associated with azoxymethane (AOM) - induced atypical crypts and /or early carcinomas. Therefore, it has been argued that BCAC, rather than ACF, should be applied as a biomarker for identifying modulators of colon carcinogenesis in a short-term experiment³². In the present study, BCAC appeared prior to the increase of adenomas and adenocarcinomas, revealing the true preneoplastic status. Our reports are in line with the conclusion drawn by Davidson³³. Since, the inhibition on the occurrence and advancement of premalignant lesions has a potential for cancer prevention, it is important to examine whether chemopreventive agents suppress the occurrence of these lesions. Our results show that GA suppressed the development of BCAC thus underlining its colon cancer chemopreventive potential.

Cell proliferation is a carefully orchestrated process and disruption of the same plays an important role in multistage carcinogenesis. Proliferating cell nuclear antigen (PCNA) can be used to mark cell proliferation activity and is helpful to study the developmental processes for the detection of dynamic changes during morphogenesis³⁴. Nucleolar organizer regions are segments of DNA that encode for ribosomal RNA. They are associated with argyrophilic proteins, and thus, they can be localized through silver staining. Quantification of argyrophilic nucleolar organizer regions proteins (AgNORs) has been considered to be an ideal technique to assess the cell cycle in paraffin wax embedded material, and previous studies have reported a direct relationship between the number of AgNORs and the number of proliferative cells³⁵. Cyclin D1 is another cell cycle regulator implicated in colon carcinogenesis³⁶. It belongs to the family of activating proteins that stimulate progression through the cell cycle. Cyclin D1 regulates the G1 to S progression of the cell cycle; it is also involved in apoptosis, in addition to its role in cell proliferation and oncogenesis³⁷. In the present study gallic acid supplementation to DMH-treated rats showed a significant decrease in the expression of cell proliferation markers viz., PCNA, AgNORs and cyclin D1 which in turn reflects the growth inhibitory effect of GA. It was demonstrated that in vitro treatment of cancer cells with GA at pharmacologically achievable and nontoxic doses ($\leq 100 \mu\text{M}$) resulted in a moderate to very strong growth inhibition, largely due to a G0/G1 arrest in cell cycle progression which correlates with the present in vivo antiproliferative activity of GA³⁸.

Furthermore the chemopreventive effect of GA on colon carcinogenesis induced by DMH in rats is evidenced by the decreased incidence and distribution of tumours along the colon. This effect of gallic acid could be associated with inhibition of cell proliferation and induction of tumour cell death.

Our results strongly suggest that though the supplementation with GA during the initiation (group 4), post-initiation (group 5) stages of carcinogenesis and the entire study period (group 6) significantly suppressed colonic neoplastic changes, the entire period treatment regimen (group 6) was found to be the most effective method of treatment as compared

to the other treatment regimens. Since colon tumorigenesis is highly sensitive to dietary intervention, patients with colonic preneoplastic lesions could markedly benefit from GA. This suggests the usefulness of the preventive agent GA for individuals at high risk for colon cancer.

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