Effect of vanadium on colonic aberrant crypt foci induced in rats by 1,2 Dimethyl hydrazine

P Suresh Kanna, CB Mahendrakumar, T Chakraborty, P Hemalatha, Pratik Banerjee, M Chatterjee

INTRODUCTION
Colon cancer is one of the most common malignancies in many regions of the world\(^1\). The idea that this cancer might be a root cause for chemoprevention stems from epidemiological evidence that some factors in the diet may play important roles in its development, where others may reduce the risk\(^{2,3}\). Experimental Colon carcinogenesis is a multistep process involving three distinct stages, initiation, that alters the molecular message of a normal cell, followed by promotion and progression that ultimately ends up with a phenotypically altered “transformed cell”\(^4\). In animal studies, treated with a carcinogen, such as, 1,2 dimethylhydrazine (DMH), methylnitrosourea, N-methyl-N′-nitro-N-nitrosoguanidine will induce colon tumors in experimental animals particularly in rodents\(^{5,6}\). Colon carcinogenesis models using DMH or the related azoxymethane, with putative preneoplastic aberrant crypt foci (ACF) as end-point marker lesions have been used to assess the influence of modulatory factors\(^7,8\).

ACF are readily discernible ‘preadenomatous’ morphological putative lesions within the colon mucosa of rodents and even in cancer patients that may contribute to the stepwise progression to colon cancer\(^9-11\). The formation and growth of ACF are associated with the induction of colon tumors in rats and are influenced by exposure to chemopreventive agents\(^{12,13}\). Natural compounds that inhibit ACF induced colon carcinogenesis have proved to be protective against colon cancer in rodents\(^{14}\).

Besides the DMH-target tissue colon, the liver was preferentially selected for assaying the biotransformation and detoxification pattern\(^{15,16}\). Many chemical changes of the liver are detectable prior to the onset of secondary pathological and nutritional changes associated with conditions such as neoplasia. The pathological alterations in the liver often act as an indicator of overall damage caused by a carcinogen since liver enzymes provide more sensitive indicators of pathogenesis than blood\(^{17}\). Efficient inactivation of both xenobiotics and endogenous toxins result in the preservation of cellular integrity and inhibition of cytotoxic events, which lead to several diseases including cancer\(^18\). Glutathione S-transferases (GST) are a family of multifunctional proteins, which act as binding proteins and also as enzymes in various detoxification processes\(^{19-21}\). GSTs have been acknowledged as preneoplastic and neoplastic markers\(^{22}\).

Cytochrome P-450s, also known as mixed function oxidases having a very broad range of substrate specificity in both exogenous and endogenous including drugs, chemical carcinogens and xenobiotics\(^{23,24}\) carry out biotransformation and reduction. Aberration in epithelial colon cell proliferation leads to hyperplasia with higher risk of colon cancers both in humans and experimental animal models\(^{25}\). Assessment of PCNA expression as an indicator of colonic cell proliferation is suggested as a putative intermediate marker of colon cancer risk\(^{26}\).

Vanadium, an element of complex chemistry is of considerable scientific and biological interest nowadays, because of its diverse physiological properties with narrow thresholds between essential and toxic doses\(^{27}\). Various studies from our laboratory have established vanadium for the very first time to be a novel biological regulator in assessing the
physiological and biochemical state of animals in a dose related manner in the detoxification of a number of xenobiotics, including electrophilic chemical carcinogens. Vanadium is observed to be capable of exhibiting some unique beneficial effects particularly, its anticarcinogenic potential under a very low dose\textsuperscript{28-30} without any adverse toxicity. Our laboratory has documented a number of works involving vanadium as a potential antineoplastic agent in rat liver carcinogenesis. Furthermore, this element has shown to be able to inhibit chromosomal and molecular damages by abating the generation of DNA single stranded-breaks and thereby maintaining the genomic integrity\textsuperscript{31}. Thus, there are good reasons to suspect that this micronutrient vanadium may be considered as a potential cancer chemopreventive agent.

In the present study, we had focused on the inhibitory effect of vanadium against the early stages of neoplastic transformation in a defined DMH-induced rat colon carcinogenesis model, since no reports from any other laboratories have documented the same, by morphometric evaluation of the ACF in colonic mucosa and colonic tumors in terms of tumor incidence, colon tumor multiplicity and tumor burden along with histological typings. Furthermore, the chemopreventive efficacy of the trace element was also investigated on certain hepatic drug metabolizing and phase II detoxifying enzyme activity patterns e.g. on liver glutathione S-transferase [GST] and Cytochrome P-450 [Cyt P-450) activities. Finally, PCNA expression as an indicator of cellular proliferation was carried out to correlate the morphometric and enzymological parameters with the protein expression to establish the possible antineoplastic potential of the said element at the cellular level.

**MATERIALS AND METHODS**

**Animals**

40 Male Sprague Dawley rats (3-4 weeks old) weighing 80-100 grams were purchased from Indian Institute of Chemical Biology (CSIR), Kolkata (Calcutta), India and quarantined for a week. They were housed 10 per cage under controlled conditions of a 12:12 hour light and dark cycle at 28±3°C. All rats were maintained on a semi purified basal diet (Lipton, Calcutta, India) and water ad libitum. All rats received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985). Body weights were recorded every 2 weeks.

**Chemicals**

1, 2 Dimethylhydrazine (DMH) and Vanadium as Ammonium monovanadate was purchased from E. Merck Ltd, Bombay, India.

**Experimental protocols**

Rats were randomly assigned into experimental and control groups. Group A: animals constituted the normal untreated controls and received saline vehicle intraperitoneally, once a week, throughout the entire course of experimental study, till 32 weeks. Group B: comprised of carcinogen control animals. 1.2 DMH was administered intraperitoneally at a dose of 20-ng/kg-body weight, once a week in 0.9 % NaCl solution (pH 7.2) for a total period of 16 weeks. Group C: included the experimental animals, which received both DMH and Vanadium treatment. Vanadium as ammonium monovanadate at a dose of 0.5-ppm was administered ad libitum through drinking water while DMH was injected in the same dose mentioned earlier for Group A animals. Vanadium treatment was initiated simultaneously with the DMH injection (termed day 0). Group D: animals were vanadium controls and received vanadium alone as ammonium monovanadate at a dose of 0.5 ppm ad libitum through drinking water. They were not subjected to any DMH injection.

Interim sacrifice was performed at 16 weeks from the day of initiation in order to evaluate the preventive efficacy of vanadium in the initial stages of carcinogenesis in terms of histology and ACF studies. The terminal sacrifice was carried out at end of 32\textsuperscript{nd} week as shown in Figure 1. All animals were fasted overnight before termination and sacrifice under light ether anesthesia. The length of the colon was recorded.

![Figure 1 Grouping and different time point of the experiment.](image)

**Histological evaluation and ACF assay**

The method of Bird\textsuperscript{32} was used to stain and highlight ACF. The number of ACF was evaluated in the 0.3 % methylene blue stained colon. ACF was scored under a light microscope with 40 times magnification to transluminate the colon. Only ACF out at end of 32 week as shown in Figure 1. All animals were fasted overnight before termination and sacrifice under light ether anesthesia. The length of the colon was recorded.

**Morphometric evaluation**

After the terminal sacrifice at 32 weeks, colons were excised from the rats, blotted dry, cut open longitudinally and the inner
surface was examined whether there were visible macroscopic lesions (neoplastic tumors). Tumors were easily discernable in the inflamed sections of the colon. The number and size of the tumors were noted for tumor incidence, multiplicity and burden studies. Tumors were classified as adenomas/ carcinomas based on the evidence of invasion through the muscularis mucosa. Those with clear-cut invasion were considered to be carcinomas while the rest were classified as adenomas\textsuperscript{[33]}. The three main axes of each macroscopic tumor from rats were measured using a vernier caliper with 0.1 mm graduation. Preneoplastic lesions were not included in tumor counts.

**Assay of GST and Cyt P-450 activities**

To determine whether vanadium could modify liver GST and Cyt P-450 activities, livers were excised immediately from all rats necropsy. The livers were perfused with saline to remove blood and minced into small pieces. Aliquots from minced livers were processed to obtain the cytosolic fraction as described\textsuperscript{[34]}\textsuperscript{[35-37]}. The activities of GST with 1,2-dichloro-4-nitrobenzene (DCNB) as substrates, and Cyt P-450 assay was determined as described\textsuperscript{[35-37]}\textsuperscript{[38]}. All assays were performed with UV-Visible Spectrophotometer (Jasco V-530). One unit of enzyme activity was the amount of enzyme catalyzing the conversion of 1 µmol of substrate to produce per min at 25 °C. Cytosolic protein concentrations were determined by the method of Lowry et al\textsuperscript{[38]} using bovine serum albumin as the standard.

**Immunohistochemistry of proliferating cell nuclear antigen (PCNA)**

Immunohistochemical staining for PCNA was performed by the avidin-biotin complex method (Sigma). Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series, immersed in 0.3 % hydrogen peroxide in absolute methanol for 30 minutes at room temperature to block endogenous peroxidase activity and then washed in phosphate-buffered saline (pH 7.2). Following incubation with normal rabbit serum at room temperature for 10 minutes to block background staining, the sections were incubated with an anti-PCNA antibody (mouse monoclonal PC 10; Sigma, USA; a 1:100 dilution) for 12 hours in a humidified chamber at room temperature. They were then reacted with 3,3’-diaminobenzidine and counterstained with Harris’ hematoxylin. For determination of PCNA-positive index, 10 full-length crypts (aberrant crypts, normal-appearing crypts or normal crypts) of each colon were examined. The number of PCNA positively stained nuclei in each crypt column was recorded. The PCNA positive index (number of positive stained nuclei X 100/total number of nuclei counted) was then calculated.

**Statistical analysis**

The statistical analysis for morphometric studies between different groups was performed by Fischer’s exact probability test for tumor incidence. Student’s t test was used to analyzed the tumor multiplicity and tumor burden.

**RESULTS**

**Food and water intake**

No appreciable change in food consumption was observed among different groups of rats. The daily food and water intakes were measured with a measuring cylinder and it was found that rats took on an average of 8-10 ml of water/day per rat.

**Mortality**

All animals survived during the entire course of the experiment.

**Body weight of rats**

Figure 2 showed the body weight of the rats in different groups sacrificed on the 32nd weeks following the first DMH injection. DMH treatment did not appreciably decrease rat body weight when compared with saline treatment, for first few weeks but by the end of the experimental study at 32nd weeks, differences between normal and carcinogen control Group A were statistically significant (P<0.05). In Group C, animals undergoing treatment with vanadium, maintained near normal body weights. Animals in vanadium control Group D displayed body weights close to those in normal Group B.

**Aberrant crypt assay**

The effect of vanadium on the growth and development of ACF, induced by DMH, in rats was given in Table 1. There was a remarkable decrease in the incidence of aberrant crypts, expressed in terms of percentage, from 100 % in carcinogen group B to 66.07 % in vanadium treatment Group C in early stages of colon carcinogenesis. The average yield of aberrant crypts for the carcinogen group was 112±3.2 ACF/Colon and the range was 90-115 ACF/Colon. For the vanadium treatment Group C, this was significantly reduced (P<0.001 when compared with Group B) to a mean value of 38±3.1 and ranged between 15-40 ACF/Colon. There were no observable foci witnessed in rats of Group D which had administration of vanadium alone ad libitum throughout the experiment.

**Histology**

Tissue sections of Group A displayed normal colonic architecture with no signs of apparent abnormality (Figure 3.1 a,b). In the Carcinogen group B, well-differentiated signs of neoplasia were evident. Nuclei were enlarged and hyper chromatic with mitosis. Simultaneously, there was a loss in nuclear polarity. Connective tissues showed edema and swelling of endothelial cells (Figure 3.2 a,b). In the vanadium treatment Group C, histology revealed no loss of nuclear polarity. Tubules were well formed while crypts lie parallel
to each other. The size and shape of the cells were uniform. Occasionally, hyper chromatic nucleus was evident. However, connective tissue invasion was not seen. No oedema or infiltration of polymorph nuclear leucocytes was sighted (Figure 3.3 a,b). There were no signs of neoplasia or toxicity observed in Group D rats administered with vanadium supplementation (Figure 4.3 a,b).

**Colonic tumor analysis**
Administration of ammonium monovanadate at the dose of 0.5 ppm, *ad libitum* through drinking water for each animal, brought about a significant reduction of tumor incidence in DMH-induced colon carcinogenesis (Table 2). In the carcinogen Group B, tumor incidence was 100% (Figure 4.1 a,b). It dropped to a significant 60% in the vanadium treated Group C (*P*<0.01 when compared to DMH control group B by Fischer’s exact probability test) (Figure 4.2 a,b). The average number of tumor (classified as adenomas, carcinomas) per tumor bearing rat was also considered in the study. Rats treated with vanadium in Group C contained significantly few colonic adenomas and carcinomas (*P*<0.05 by Student’s *t* test) compared to rats administered with DMH only (Table 2). In addition, a significant reduction (*P*<0.05) in colon tumor burden (sum of tumor sizes per animal) was also evident in Group C when compared to those in carcinogen control Group B. The results were statistically significant (Table 3). There were no marked changes observed in Group D (Figure 4.3 a,b).

**Figure 3** 3.1 (a): showing the normal colonic architecture view of rats sacrificed at end of week 16 (Low power). 3.1 (b): showing the normal colonic architecture view of rats sacrificed at end of week 16 (High power). 3.2 (a): representing the carcinogen-induced group B rats sacrificed at end of week 16 (Low power). 3.2 (b): representing the carcinogen-induced group B rats sacrificed at end of week 16 (High power). 3.3 (a): showing the treatment with vanadium group C rats sacrificed at the end of week 16 (Low power). 3.3 (b): showing the treatment with vanadium group C rats sacrificed at the end of week 16 (High power).
Figure 4 4.1 (a): showing the carcinogen-induced group B rats sacrificed at end of week 32 (Low power).
4.1 (b): showing the carcinogen-induced group B rats sacrificed at end of week 32 (High power).
4.2 (a): showing the treatment with vanadium group C rats sacrificed at the end of week 32 (Low power).
4.2 (b): showing the treatment with vanadium group C rats sacrificed at the end of week 32 (High power).
4.3 (a): showing the vanadium control group D rats with no signs of toxicity sacrificed at the end of week 32 (Low power).
4.3 (b): showing the vanadium control group D rats with no signs of toxicity sacrificed at the end of week 32 (High power).

Table 2 Chemopreventive efficacy of 0.5-ppm vanadium (supplemented ad libitum through drinking water) on the incidence and multiplicity of DMH-induced rat colonic tumors

| Group | Number of rats | Colon tumor incidence (percentage of tumor-bearing rats) | Total number of tumors | Colon tumor multiplicity (mean tumor/animal, Mean±SD) |
|-------|----------------|---------------------------------------------------------|------------------------|-----------------------------------------------------|
|       | Total          | Tumor counts                                           | Adenoma                | Carcinoma   | All neoplasia | Adenoma | Carcinoma | All neoplasia |
| A     | 10             | -                                                       | -                      | -          | -            | -       | -         | -            |
| B     | 10             | 10                                                      | 100                    | 97         | 64           | 120     | 9.7±0.3   | 6.4±0.1      | 12±0.1       |
| C     | 10             | 06                                                      | 60a                    | 23         | 16           | 31      | 2.3±0.2   | 1.6±0.2      | 3.1±0.1b     |
| D     | 10             | -                                                       | -                      | -          | -            | -       | -         | -            |

aP <0.01 vs DMH-only group B by Fischer’s exact probability test; bP <0.05 vs Group B by Student’s t-test.
As there is strong correlation between ACF formation and colon carcinogenesis, the observation amply imply that supplementation by 0.5-ppm vanadium under the conditions of the experiment, can greatly affect the post initiation stages of colon carcinogenesis by altering the efficacy at which DMH can initiate foci appearance. Increased mitotic activity, which have been proposed as a biomarker of the early stages of colon cancer[40] was observed in most of the ACF induced by DMH administration alone. Treatment with vanadium greatly restored normalcy in the colonic epithelial cells. The ability of vanadium to reduce the number of ACF per colon also indicates that the anti-carcinogenic potential of vanadium could be mediated through an enhanced repair or remodeling of preneoplastic lesions[41].

In our observation, we have studied vanadium mediated inhibition of the tumor multiplicity coupled with tumor burden as a percentage of the colonic length. This observation is of interest if one considers that there was no major difference in body weights among the normal rats and rats in Group C. This is particularly important because nutritional deprivation causing body weight loss may parallel a decrease in tumor burden[42]. The variation in weight gain among the different groups under experiment, thus, do not seem to be significant when evaluating possible causes for the observed differences in the induction of AC or tumors.

Treatment of rats with drinking water supplemented with vanadium for 16 and 32 weeks not only decreased the number of preneoplastic foci but also caused a decrement in the tumor incidence/tumor multiplicity with a concomitant reduction in tumor burden as a percentage of colonic length. This strongly suggests the potentiality of vanadium in inhibiting/slowing tumorigenesis in the rat colon.

Phase II enzymes help to inhibit the formation of electrophiles and catalyze their conversion to inactivate conjugates making them more water soluble and readily excretable from the cell. It is the cellular balance between the Phase I activating enzymes and Phase II detoxifying enzymes that contribute to one’s risk of developing cancer[43]. GSTs catalyze the reaction of the compounds with thiol group of GSH by itself or together with GST can function as a non-critical antioxidant as well as biotransformative mechanism. Cellular GSH by itself or together with GST can function as a non-critical nucleophile for conjugation reactions and play an important role in the inactivation of electrophilic compounds[44]. Therefore, an elevation of GSH level indicates an increase in the systemic ability to detoxify electrophilic compounds including carcinogens. Data from several laboratories continue to suggest a relationship between decreased GST expression and an increased risk for cancer[47-49]. The decrease may be associated further with interference of protein synthesis and accumulation of electrophilic metabolites. Increased GST level towards normal value clearly indicates that the tumor genesis burden.

**DISCUSSION**

Variable inhibitory effects of vanadium on the incidence of preneoplastic lesions (ACF) were observed during different phases of colorectal carcinogenesis. The finding of the histological and morphometric study clearly supports that trace element vanadium holds a promising anticancer potential with respect to colon carcinogenesis[39]. The results suggest that chemically induced carcinogenesis in the rat colon follows a distinct pathway where histogenesis obeys the ACF-adenoma-carcinoma sequence in the mid and distal colon and the ACF are an intermediate stage only existed in better-differentiated tumors. A linear relationship between AC formation and colon tumor induction for the same group of laboratory animals could also be established.

**Liver GST and Cyt P-450 activities**

Liver GST and Cyt P-450 activities at the end of the study were shown in Table 4. DMH treatment in Group B significantly elevated liver GST (P<0.05) and Cyt P-450 (P<0.001) activities using CDNB as a substrate when compared with those of Group A. GST activities in Group C was significantly greater than those in Group B (P<0.02) and Cyt P-450 activities in Group C was also significantly greater than those in Group B (P<0.001).

**Table 4** Liver GST and Cyt P-450 activities (mean ±SE, n=10)

| Group               | Cyt P-450 activity (mU/mg protein) | GST-CDNB (mU/mg protein) |
|---------------------|-----------------------------------|--------------------------|
| A Normal Control    | 0.62±0.06                         | 0.83±0.10                |
| B DMH-Control       | 0.20±0.03c                         | 0.45±0.03c               |
| C DMH + V           | 0.41±0.01c                         | 1.98±0.09f               |
| D V-Control         | 0.47±0.05                          | 0.90±0.19                |

\( \text{P} <0.001 \) vs Group A, \( \text{P} <0.05 \) vs Group A, \( \text{P} <0.001 \) vs Group B, \( \text{P} <0.02 \) vs Group B.

**PCNA-labeling index in ACF**

The PCNA-labeling indices in ACF were shown in Table 5. The mean PCNA-labeling indices in ACF of Group C were significantly lower than that of Group B (P<0.005 and P<0.005, respectively).

**Table 5** PCNA-labeling index of colonic mucosa of rats treated with DMH, supplemented with vanadium (V) 0.5-ppm ad libitum in drinking water (means ±SD)

| Group               | No of rats | ACF(N numbers of ACF or crypts) |
|---------------------|------------|---------------------------------|
| A Normal control    | 10         | -                               |
| B DMH control       | 10         | 35±5 (10)                       |
| C DMH + V           | 10         | 23.6±2 (10)                     |
| D V-Control         | 10         | -                               |

\( \text{P} <0.005 \) vs Group B by Student’’s t-test.
is not high, at the same time shows that vanadium is a good protective agent against DMH induced colon carcinogenesis.

Preliminary studies from our laboratory[50,51] have shown that under a certain optimum dose of 0.5 ppm, the trace element could lead to stable induction of GST activity without any apparent signs of toxicity. A probable mechanistic explanation could be increased transcription of GST gene and/or allosteric modification of the enzyme. Alternatively, this increase in GST activity by vanadium can be viewed as the host cellular response in boosting the GSH-related conjugation system against the possible free radical mediated stress.

In the present study, we also report that vanadium functions as an anticarcinogen by altering the activity of Cyt P-450 related enzyme. Vanadium might have induced the Cyt P-450 level due to its property as a heavy metal. Since the dose used is non-toxic[52], it is having a positive action on inhibiting tumor promotion. The induction of Cyt P-450 may also be due to alteration of the ATP/ADP ratio by the inhibition of oxidative phosphorylation thereby increasing the NADPH content rapidly for the mixed function oxidase system to act. Alternatively, vanadium may elevate the Cyt P-450 level by regulating the transcriptional activation of the P-450 gene[53]. Considering the relative persistence of oxidative damage, antioxidant defense and biotransformation alterations, we could predict that the biochemical markers measured in the liver may well be a prognostic marker of the distant neoplasm of the colon, even at the early stages of preneoplasia at 16 weeks.

Finally, PCNA-labeling index, an intermediate biomarker of carcinogenesis, was decreased in DMH-treated ACF by supplementation of vanadium in the drinking water. Cell proliferation plays an important role in multistage carcinogenesis with multiple genetic changes[54]. PCNA is an auxiliary protein of the DNA polymerase delta, reaching an expression peak during the S-phase of the cell cycle and playing an important role in cellular proliferation[55]. PCNA has been used as an intermediate biomarker in chemoprevention of colorectal cancer[56]. Zheng et al[57] observed that Vitamin A significantly decreased PCNA in the AOM-induced colorectal cancer animal model. Thus, the inhibitory effect of vanadium may be due, in part, to modification of cell proliferation through the above mechanisms.

One of the predominating factors which often limit the therapeutic efficacy of many antineoplastic elements and their complexes is their considerable toxic side effects associated with hepato and nephrotoxicity. However in the present study, supplementing vanadium at 0.5 ppm have shown no clinical signs of toxicity such as decrease in food and water intake, retarded growth or eventual death.

In conclusion, the results of this study suggest that daily supplementation of 0.5 ppm vanadium in the form of ammonium monovanadate in the drinking water has a positive beneficial effect against chemically induced colonic preneoplastic progression in rats induced by DMH, which provides an effective dietary chemopreventive approach to disease management. However, other definitive bioassay including protein expression and documentation of specific molecular markers is now being planned in our laboratory to establish the surrogate end-point biomarker in vanadium-mediated cancer chemoprevention.

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