



## DIETARY PHYTATE LOWERS IRON STATUS, SUPPRESSES ABERRANT CRYPT FOCI AND INHIBITS PROGRESSION TO ADENOMA IN AZOXYMETHANE-INDUCED COLON CANCER

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### ABSTRACT

Phytate is known for its anti-neoplastic activity in various cancers, but the underlying mechanisms are less understood. We therefore examined pure Inositol Hexaphosphate (IP<sub>6</sub>, phytate) as a dietary ingredient provided post-initiation, for its ability to inhibit dysplastic aberrant crypt foci (ACF) formation, and their progression to colon adenoma/adenocarcinomas viz. a viz. the role of altered iron homeostasis, in F344 rats using azoxymethane (AOM) as a carcinogen. Treatment with 1 or 2% IP<sub>6</sub> was initiated after 20 weeks of AOM administration and continued for 16 weeks. IP<sub>6</sub> suppressed ACF formation and inhibited their progression to tumors in a dose-dependent manner. Phytate significantly ameliorated the tumor and inflammatory markers such as proliferated cell nuclear antigen (PCNA),  $\beta$ -catenin, cyclooxygenase-2 (COX-2), whose expression was upregulated with azoxymethane. Systemic iron status indicators including hemoglobin, serum iron, ferritin, and hepcidin levels suggested a clear depletion of body iron stores with phytate supplementation. Apoptotic indices, such as TUNEL staining and caspase-3 activity were promoted by IP<sub>6</sub> in AOM-administered colon epithelium. Thus, dietary phytate consumption effectively reduced the tumor incidence and multiplicity even in a post-initiation model of azoxymethane, with a decrease in body iron stores.

### KEY WORDS

Inositol Hexaphosphate; azoxymethane; iron; proliferation; apoptosis; colon

### INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of morbidity and mortality (Ferlay et al. 2013). The incidence of colon cancer in developed countries of Asia is far more than the United States and other Western European countries. Recent estimates of CRC in India indicate a marginal increase, making it the fifth most common cause of cancer mortality among Indian men and women (Ferlay et al. 2013; Mallath et al. 2014). The etiology and molecular pathogenesis of colorectal cancer is complex and multifactorial (Fearon 2011), with inherited mutations, environmental

interactions, and dietary nutrients playing a key role in the development, progression and inhibition of colorectal cancer (Qiao and Feng 2013).

Iron is an essential micronutrient required for various cellular processes, but excess iron has been recognized as a risk factor for colon cancer (Chua et al. 2010). A recent meta-analysis study also supports a positive dose-response correlation of dietary iron levels with CRC risk (Qiao and Feng 2013). Multiple epidemiological studies have shown a positive correlation between iron and colorectal cancer, including dietary iron consumption and total body iron stores (Wurzelmann et

al. 1996). On the other hand, some studies have reported no association between the risk of colorectal cancer and body iron levels or dietary iron uptake (Herrinton et al. 1995). Iron supplementation or serum ferritin concentration was not linked to the recurrence of colorectal adenoma (Tseng et al. 1997). The discrepancy between colon cancer risk and dietary iron appears to depend on the metabolic iron requirements and iron absorption rates. With only 10% of the ingested iron being absorbed in the small intestine, unabsorbed iron tends to accumulate in the colon lumen (Shaheen et al. 2003). Increased tumor frequency and multiplicity observed with feeding of high iron diets, during induction of carcinogenesis in animal models implicates the adverse effects of unabsorbed iron and its interactions with carcinogens (Seril et al. 2002). Given the essential role of iron in cell proliferation and viability, it is reasonable to argue that altered iron homeostasis, decreased intake of iron and decreased transferrin saturation may aid in the suppression of colon cancer and that modulation of iron homeostasis by natural means can prove to be an effective chemoprotective strategy.

Dietary fiber, of which phytate is a part, has been well studied for its protective effect in human colorectal cancer (Nelson 1992). In this regard, IP<sub>6</sub> was shown to downregulate PCNA, a proliferation marker and inhibit growth of HT-29 human colon cancer cells in a dose- and time-dependent manner (Yang and Shamsuddin 1995). Continued phytate supplementation during azoxymethane administration effectively reduced the pre-neoplastic aberrant crypt foci formation in F344 rats (Pretlow et al. 1992). Inositol Hexaphosphate was supplemented in drinking water even before or concomitant with the carcinogen administration, resulting in reduced tumor burden (Shamsuddin et al. 1988; Ullah and Shamsuddin 1990). The anti-neoplastic activity of phytate has been attributed to altered gene function (Bode and Dong 2000), cell cycle inhibition (Shamsuddin et al. 1997), modulation of cellular signal transduction (Dong et al. 1999), and antioxidant function (Graf and Eaton 1990). Phytate is originally regarded as an anti-nutrient, due to its mineral chelating ability. However, the anti-nutrient function of dietary phytate and its ability to modulate iron homeostasis has not been examined in detail, given the relationship between dietary iron and colon cancer. This study is more relevant considering the fractional iron absorption

and high levels of unabsorbed luminal iron interacting with the colon epithelium.

We have therefore used 1 or 2% dietary phytate supplementation for 16 weeks after azoxymethane administration in male F344 rats, to understand if pure phytate provided inherently as a dietary ingredient decreases the colon cancer development and progression by altering iron homeostasis.

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## MATERIALS AND METHODS

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### **Chemicals and reagents**

Azoxymethane, Inositol Hexaphosphate Na salt and 10% (v/v) neutral buffered formalin were purchased from Sigma (St. Louis, MO, USA). ApoptTag Peroxidase *in situ* apoptosis detection kit was purchased from Millipore. All other chemicals and reagents used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

### **Diet and animals**

This study was performed according to the guidelines approved by the Institutional Animal Care and Ethics Committee (IACEC) of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad. A total of 48, five-week-old male Fisher 344 rats (*Rattus norvegicus*), weighing approximately 90–100 grams were housed individually in plastic cages with wood-chip bedding. The animals were acclimatized for 1 week and fed the American Institute of Nutrition (AIN-93G) diet *ad libitum* (Bodiga et al. 2012). They were housed in a well-ventilated room at approximately 25 to 27°C, with 50 ± 10% relative humidity, and a 12-hour light/dark cycle. Hygienic conditions were maintained with weekly changes of woodchip beds. The rats were randomly assigned to control (n = 16) or AOM (n=32) groups. AOM group received azoxymethane intraperitoneally (15 mg/kg body weight) diluted in 0.9% (v/v) saline, once weekly for two successive weeks (Bird 1987). Control group received an equal volume of saline and served as the vehicle control. After 20 weeks of initiation, 8 animals from each group were sacrificed to monitor the formation of aberrant crypt foci (ACF) in colon. The remaining animals (n=24) in AOM group were randomly divided into 3 groups and fed either 0, 1 or 2% phytate containing AIN-93G diet for the next 16 weeks (n =8, each). Phytate was added at the expense of corn starch in AIN-93G diet. The effect of dietary phytate on suppression of colonic ACF and their progression into colonic tumors was studied after 16 weeks of phytate

consumption. Food consumption was recorded daily, and the body weights were recorded weekly throughout the study. Aliquots of blood were obtained with EDTA as anticoagulant to measure the hematological parameters. The remaining blood was collected in a plain tube without anticoagulant and the serum was separated for subsequent analysis of Fe, ferritin, total Fe-binding capacity (TIBC) and hepcidin. The livers and colonic mucosa were collected, washed with ice-cold saline solution (0.9% NaCl, wt/vol), weighed, snap frozen in liquid nitrogen, and stored immediately at  $-80^{\circ}\text{C}$  until further analysis.

#### **Iron concentrations in diet and liver**

Samples of diet and liver (1 g of dry weight) were mineralized following the method described previously (Sreedhar et al. 2004). Iron concentrations were determined by atomic absorption spectrophotometry (Shimadzu AA7000).

#### **Hematological indicators**

Hemoglobin and hematocrit concentrations were determined using a hematology analyzer Sysmex K-1000D (Sysmex Corp., Tokyo, Japan).

#### **Serum Ferritin**

Serum ferritin concentration was determined using the Rat Ferritin ELISA Kit (BioVendor GmbH, Heidelberg, Germany). The absorbance of the reaction was read at 450 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA).

#### **Serum Fe, TIBC, and Transferrin Saturation**

To calculate the rate of transferrin saturation, serum Fe concentration and TIBC were determined using Sigma Diagnostics Iron and TIBC reagents (Sigma-Aldrich). The absorbance was read at 550 nm on a microplate reader (Bio-Rad Laboratories Inc.). The percentage of transferrin saturation was calculated from the following equation:

$$\begin{aligned} \text{Transferrin saturation (\%)} \\ = \frac{\text{Serum Fe Concentration } \left(\frac{\mu\text{g}}{\text{L}}\right)}{\text{TIBC } \left(\frac{\mu\text{g}}{\text{L}}\right)} \times 100 \end{aligned}$$

#### **Serum Hepcidin**

Hepcidin-25 concentrations were determined using a DRG ELISA Kit (DRG Instruments GmbH, Marburg, Germany). The microtitre wells were coated with a monoclonal (mouse) antibody directed towards an antigenic site of the hepcidin-25 molecule. Endogenous hepcidin-25 in the sample competes with hepcidin-25-biotin conjugate for binding to the coated antibody. After incubation, the unbound conjugate was washed

off and a streptavidin-peroxidase enzyme complex was added to each well. Substrate was added to detect antigen-antibody-enzyme complex and development of blue color. The microplate was read at 450 nm and the intensity of color developed was inversely proportional to the concentration of endogenous hepcidin in the sample. Results were expressed in nanograms per milliliter of serum.

#### **ACF analysis and tumor assessment**

ACF analysis and tumor incidence was assessed according to Bird (Bird 1987), after 20 and 36 weeks of AOM administration. The colons were longitudinally opened, rinsed with saline and fixed flat between two sheets of filter paper in 10% buffered formalin for at least 24 h. The colons were then cut into 2-cm segments and stained with 0.2% methylene blue in Krebs-Ringer solution for 10 min and were then placed mucosal side up on a microscope slide and observed through a light microscope at 400 x magnification. Crypts with increased size, increased separation from lamina to basal surface, and appearance of pericryptal zone were distinguished as aberrant crypts. Number of crypts in each focus determined the crypt multiplicity. For tumor assessment at the end of 36 weeks, colon tissue was removed, dissected longitudinally, flushed with phosphate-buffered saline (PBS), fixed in 10% (v/v) neutral buffered formalin prior to staining with hematoxylin and eosin (H&E). The tumor incidence was described as the percentage of total animals with adenoma/adenocarcinoma, while tumor multiplicity was defined as the average number of tumors per tumor-bearing rat. The number and size of the colon tumors present was also recorded.

#### **Apoptosis assay**

*In situ* nick-end labeling (TUNEL) of fragmented DNA was performed on paraffin embedded sections (4-5  $\mu\text{m}$ ) using the manufacturer's protocol (ApoTag Peroxidase *In Situ* Apoptosis Detection Kit, Chemi-Con International). The percentage of positive apoptotic cells was calculated after brown and green nuclei were counted by an operator who was blinded to the treatments of each sample (Bodiga et al. 2005). Values represent the mean  $\pm$  standard error of the mean (SEM) from at least 5 different sections.

#### **Western blotting**

Cytoplasmic, membrane, and nuclear fractions were isolated from colonic mucosa scraped from the serosal layers using the NE-PER nuclear and cytoplasmic

extraction kit from Thermo Scientific. 30 µg of protein were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies against β-catenin (Cell signaling, Cat No. 8480), cox-2 (Cell signaling, Cat No. 4842), lamin B1 (abcam, ab16048), GAPDH (abcam, ab9485) overnight at 4°C. Primary antibody binding was detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:3,000; Cat No. A0208; Beyotime Institute of Biotechnology) and was visualized using an enhanced chemiluminescence (ECL) detection system (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA). Band intensities were quantified using the software (Bio-Rad) and the relative expression with respect to internal controls GAPDH, Lamin B1 has been represented in the graphs.

#### **Statistical analysis**

Statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 17.0. The data was expressed as the mean ± standard deviation (SD) and analyzed using a one-way analysis of variance (ANOVA). A *P*-value < 0.05 was considered significant.

## **RESULTS**

### **Body weight**

Food intake did not differ significantly across the groups throughout the experimental duration. The body weights of all groups of rats increased continuously throughout the duration of the study and were not significantly different from each other (data not shown). The absence of significant difference in body weight with phytate supplementation indicates that the amount of phytic acid provided in the diet was well-tolerated by the animals.

### **ACF analysis**

All the rats treated with AOM developed ACF, whereas no evidence of ACF could be detected in the colons of vehicle-treated control animals, at 20 weeks after treatment with AOM. Crypt multiplicity analysis of ACF indicated that all segments of the large intestine showed similar number and incidence of crypts per focus. Normal mucosa showed crypts arranged in

parallel with round nuclei and mucin stained blue (Figure 1A), whereas hyperplastic aberrant crypt foci showed mucin depletion and elongated nuclei (Figure 1B). The data on the modifying efficacy of dietary phytate intervention on further development of colonic ACF and their progression into colonic tumors was studied at 36 weeks, i.e., after 16 weeks of supplementation with different levels of dietary phytate (1 or 2%). The numbers and incidence of ACF, along with presence of non-invasive and invasive colon tumors is presented in the Table 1. Representative images of normal and aberrant crypt stained with methylene blue, adenoma with atypical hyperplasia, adenocarcinoma, and invasive adenocarcinoma, observed in AOM-administered animals is shown in Figure 1 (C-H). Adenoma is defined as a benign colonic tumor, with proliferation of the colonic gland lined with neoplastic colonic epithelium, whereas adenocarcinoma is a malignant colonic tumor consisting of invasive glands lined with pleomorphic hyperchromatic epithelium. The mean number of ACF per entire colon in the AOM administered animals was 155±14. Dietary administration of phytate for 16 weeks, after initiation with AOM significantly (*P*<0.001) reduced the number of ACF to 87 and 59 with 1% and 2% phytate, respectively. Furthermore, the number of ACF consisting of >4 crypts also decreased significantly (*P*<0.05) in phytate-fed rats as compared to AOM alone-treated rats. The tumors were classified as invasive, noninvasive, differentiated or poorly differentiated by a pathologist, who was blinded to the study. A total of 8 tumors were observed in the AOM-alone administered animals, which included both invasive (37.5%) and non-invasive (62.5%) tumors. Two animals in AOM-administered and 1% phytate-fed group showed non-invasive tumors. A significant reduction in tumor multiplicity was observed in the group that received 2% phytate. Further, preneoplastic, dysplastic ACF number and incidence decreased significantly in both 1 and 2% phytate fed animals compared to AOM-administered, no-phytate fed animals. In order to substantiate these results, we determined the other tissue markers of proliferation by immunoblotting.

**Table 1. Inhibitory effect of phytate on AOM-induced aberrant crypt foci and tumor incidence in Fisher 344 male rat colon.**

Treatment	Incidence of ACF (%)	ACF/colon	Crypt multiplicity of ACF				Tumor incidence	
			1 crypt	2 crypts	3 crypts	≥4 crypts	Non-invasive (%)	Invasive (%)
Control	0/8 (0)	—	—	—	—	—	—	—
AOM	8/8 (100)	155± 14	52±1	65±8	22±6	16±6	5 (62.5)	3(37.5)
AOM + 1% Phytate	8/8 (100)	87 ± 9*	33±3*	26± 2*	16± 2*	12 ± 2	2 (100)	—
AOM + 2% Phytate	8/8 (100)	59 ± 8*	21±2*	18± 3*	12± 3*	8 ± 2	—	—

NOTE: Data are shown as mean ± SEM of eight samples in each group

\* $P < 0.001$ , vs. AOM

**Table 2. Apoptotic index in colonic mucosa of phytate-treated AOM-induced colon cancer**

Treatment	Apoptotic cells (%)
Control	3.28 ± 1.04
AOM	2.58 ± 1.12
AOM + 1% phytate	22.50* ± 5.60
AOM + 2% phytate	36.86 **± 6.64

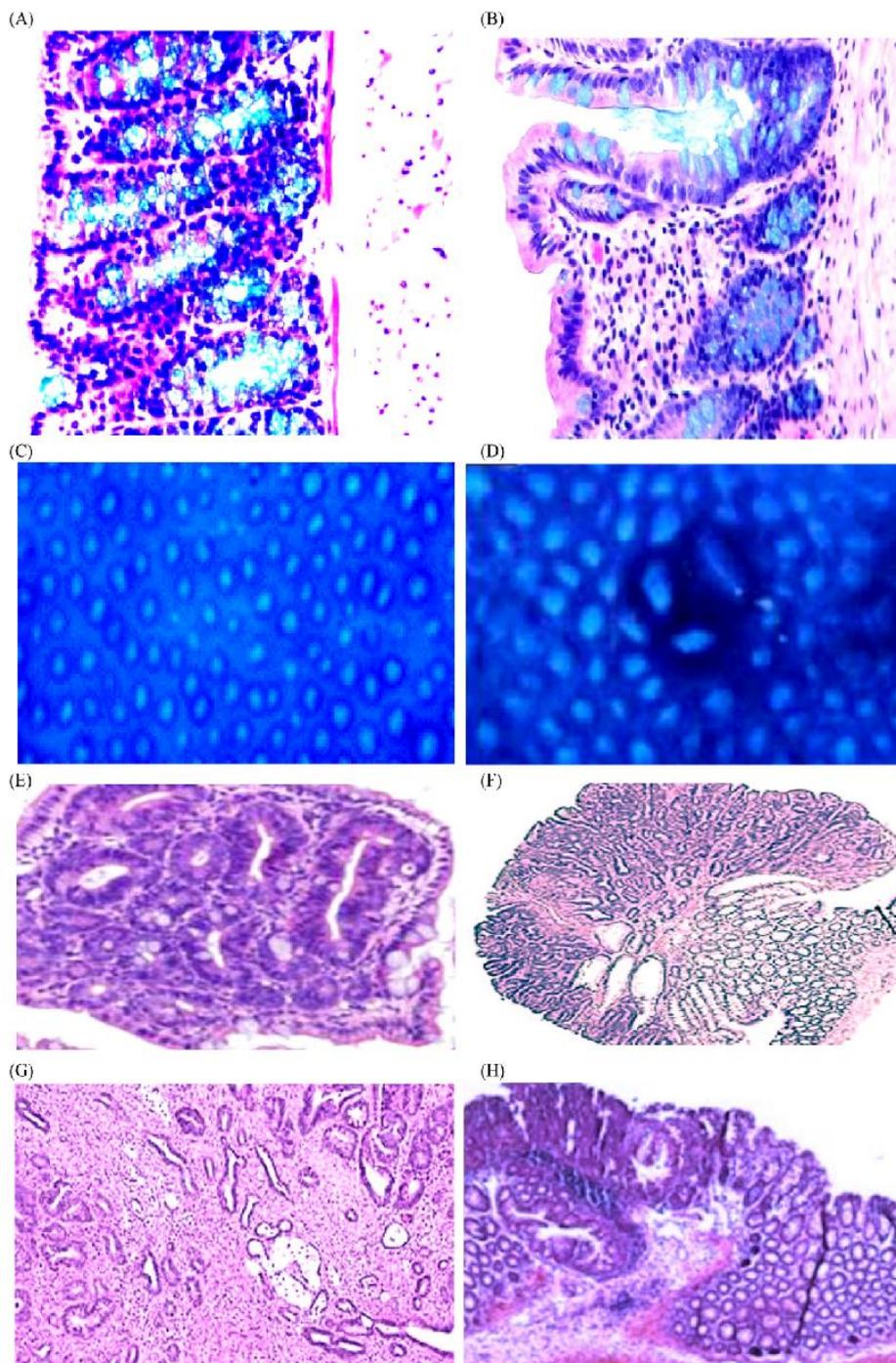
Each value is expressed as mean ± SEM of three determinations. Values in the same column different superscripts indicates significant difference by Tukey's test ( $P < 0.05$ ). Administration of 1% and 2% phytate significantly increased the apoptotic cells compared to the AOM-alone group ( $P < 0.05$ ).

**Table 3. Iron status indices in AOM-administered and phytate-fed rats**

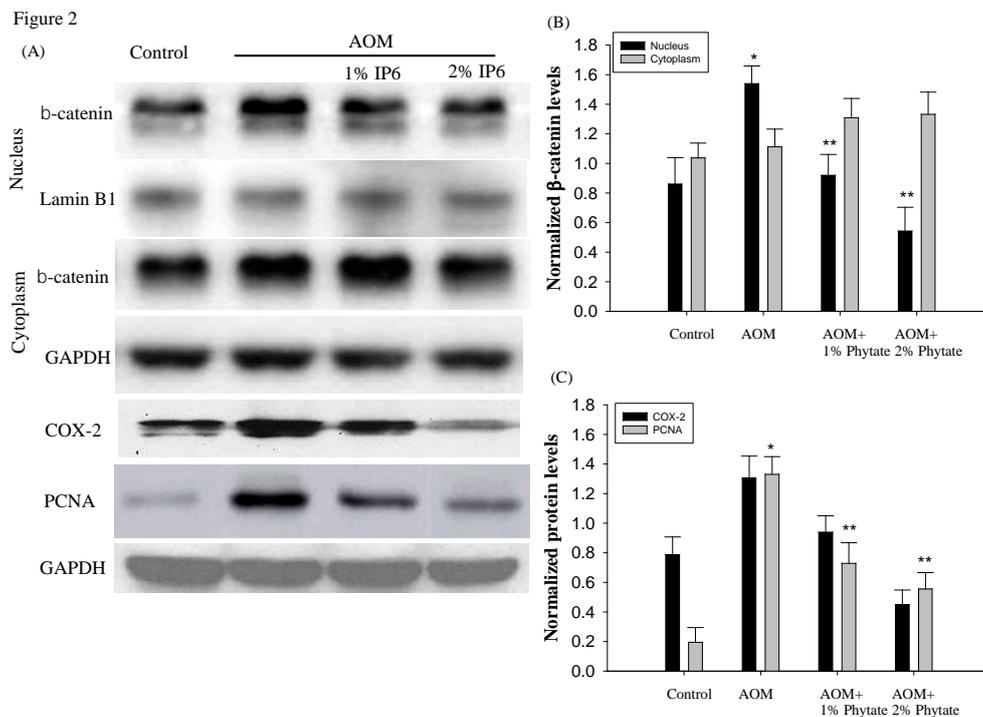
	Control	AOM	AOM+1% Phytate	AOM+2% Phytate
Hb (g/dL)	15.60±1.24	14.68±1.36	10.2*±1.80	7.46**±1.20
Hct, %	40.2±0.68	39.4±0.60	30.6*±0.50	24.0**±1.54
Serum iron (µg/dL)	200.8±20.3	198.5±17.8	122.4*±34.8	56.8**±8.5
TIBC (µg/dL)	600.0±70.8	602.8±60.4	681.2*±60.2	758.0**±75.5
Liver iron (µg/g wet tissue)	38.0±7.16	35±3.48	28.0*±2.20	20.4**±1.80
Serum ferritin (ng/mL)	1480.8±65.0	1468.2±60.8	1120.5*±58.0	850.4**±42.8
Serum hepcidin (ng/mL)	18.25±0.50	17.6±0.62	13.4*±0.46	12.2*±0.54

Hb- hemoglobin; Hct- hematocrit, TIBC- total iron binding capacity. Data is expressed as mean ± SEM of at least 6 determinations. Value in the same row with different superscript letter indicates significant difference by Tukey's test  $P < 0.05$ . Administration of 1% and 2% phytate significantly decreased the iron status compared to the AOM-alone group ( $P < 0.05$ ).

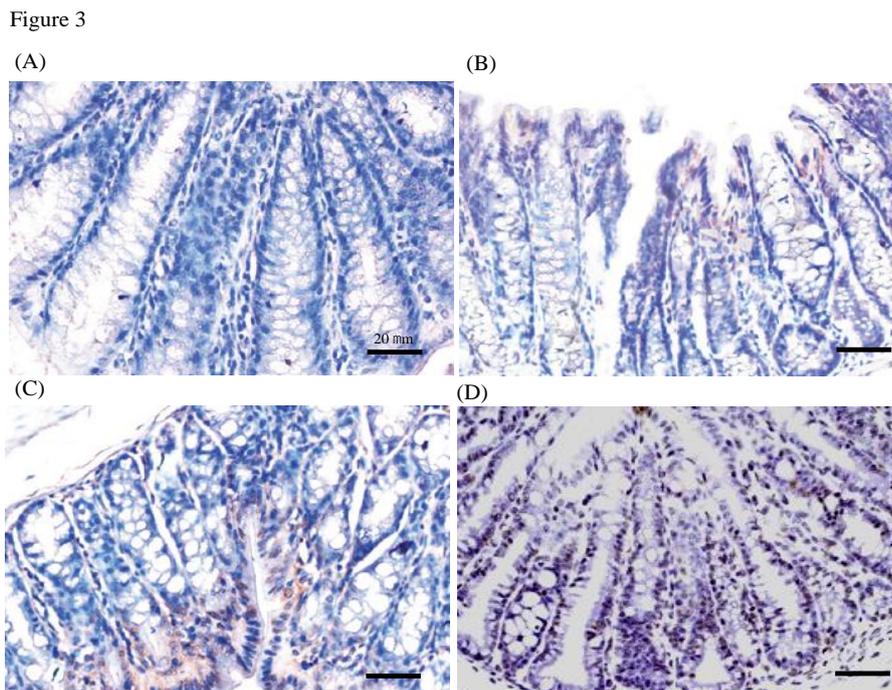
**Figure 1. Normal rat colorectal mucosa (A) with crypts arranged in parallel and mucin stained blue using trichrome stain. Normal colon epithelial cells exhibit rounded nuclei. (B) represents hyperplastic aberrant crypt foci (ACF) from azoxymethane-injected rats showing mucin depletion and elongated nuclei. (C) represents methylene blue stained normal crypts. (D) represents aberrant crypt foci. (E-H) represent adenoma, adenoma with atypical hyperplasia, adenocarcinoma and invasive adenocarcinoma, respectively observed in AOM-administered animals.**



**Figure 2. Western blot analysis of  $\beta$ -catenin, COX-2 and PCNA in colon epithelium. (A) Representative images of immunoblots of  $\beta$ -catenin in nuclear and cytoplasmic portion along with respective loading controls lamin B1 and GAPDH is shown. Also shown are changes in COX-2 and PCNA protein levels across groups. (B) Quantitative analysis of  $\beta$ -catenin protein expression in nuclear and cytoplasmic compartments; Bars with error bars indicate mean  $\pm$  SE from 6 observations; \*indicates  $P < 0.05$  vs. control; \*\* indicates  $P < 0.001$  vs. AOM (C) Quantitative analysis of COX-2 and PCNA protein expression; Bars with error bars indicate mean  $\pm$  SE from 6 observations; \* indicates  $P < 0.05$  vs. control; \*\* indicates  $P < 0.001$  vs. AOM.**



**Figure 3. Representative images showing TUNEL staining in colon of control (A) and AOM-injected rats (B-D), supplemented with 1% phytate (C) or 2% phytate (D) for 16 weeks post-initiation ( $\times 40$  objective, scale bar = 20  $\mu$ m).**



### **Expression of $\beta$ -catenin, COX-2 and PCNA in colon tissue**

Tissue markers of altered signaling pertaining to enhanced proliferation and increased inflammation were studied in the tissue homogenates by immunoblotting (Figure 2).  $\beta$ -catenin in the colonic mucosa was barely detectable in the control animals. In the AOM-alone group,  $\beta$ -catenin protein was more intense and detected both in the cytoplasmic and nuclear portion of epithelial cells. Relatively high nuclear accumulation of  $\beta$ -catenin observed in AOM-administered animals was found to be lowered with phytate supplementation (Figure 2). Decrease in nuclear  $\beta$ -catenin was more with 2% than with 1% phytate. Because phytate suppressed  $\beta$ -catenin expression, the susceptibility of colon tumors to phytate might also be due to inhibition of the Wnt signaling pathway via the suppression of COX-2 expression. Thus, the expression of COX-2 during colon carcinogenesis was investigated to determine whether phytate can modulate COX-2 expression. Overexpression of inflammatory markers is a hallmark in colorectal tumors (Paul et al. 2010). In addition to the effects on  $\beta$ -catenin, our current data revealed that colon tissue from the control group exhibited a low level of COX-2 immunoreactivity. AOM-administration resulted in a significant increase in COX-2 expression compared to control animals. Incorporation of dietary phytate (both 1% and 2%) resulted in significant decrease of COX-2 expression, indicating lowered inflammation.

Lower COX-2 expression in rats indicated that phytate might be associated with the suppression of cell proliferation and induction of apoptosis, which may contribute to a decrease in the colon tumor incidence and multiplicity (adenomas and adenocarcinomas). Thus, the effect of treatment with phytate on the cell proliferation marker, PCNA in the AOM-injected rats was examined. Western blotting analysis of PCNA clearly showed a strong intensity in AOM-treated colon tissue samples and its reduction following phytate treatment, as shown in a representative Western blot (Figure 2A). The quantitative analyses of PCNA-expression in different groups (Figure 2B) clearly showed a significant  $P < 0.001$  increase in PCNA-expression from the colonic mucosa of AOM-treated rats compared to control rats; both groups received AIN-93G control diet alone. The experimental diets containing different levels of phytate (1 or 2%) given

post-initiation significantly ( $P < 0.001$ ) decreased the PCNA concentrations in the colonic mucosa of AOM-treated rats.

### **Phytate induces apoptosis in colonic tumors**

Normally, the growth rate of preneoplastic or neoplastic cells exceeds that of normal cells, due to dysregulation of the cell-growth and cell-death machinery (Khan et al. 2007). Thus, colon tissue sections were evaluated for TUNEL staining, to examine the presence of apoptotic cells. TUNEL staining showed a large number of apoptotic cells (brown staining) in phytate-supplemented groups compared to the AOM-alone group (Figure 3). Quantitative analysis of the brown stained nuclei in different groups, indicating the apoptotic index is shown in Table 2. In the AOM-alone group, the mean number of apoptotic cells was 2.58%, as compared to 3.28% in control animals. Analysis also revealed that the supplementation of phytate in AOM-induced rat colorectal cancer resulted in a dose-dependent increase in apoptotic cells, as shown by an increase in brown nuclei (Figure 3). Thus, supplementation of phytate resulted in increase of apoptotic cells.

### **Dietary phytate alters iron status**

To understand if dietary phytate modified the iron status of AOM-administered animals, we assessed various iron status indicators shown in Table 3. At the end of the study, vehicle (control) and AOM-administered rats showed a similar profile of hematological parameters, with no significant differences. The mean hemoglobin levels, hematocrit, total iron binding capacity (TIBC), liver iron, serum ferritin and hepcidin levels were comparable between control and AOM groups. However, consumption of 1% and 2% phytate in AOM-administered rats resulted in a drastic difference in iron status indices compared to AOM ( $P < 0.001$ ), suggesting a significant depletion of circulating iron and body iron status with phytate supplementation. Hemoglobin levels and hematocrit dropped significantly in AOM + 1 or 2% phytate-fed rats. Serum iron levels decreased significantly in 1% phytate-fed rats, but more so in 2% phytate-fed rats. Expression levels of ferritin and hepcidin were found to positively correlate with other iron status indicators, which are increased in response to increased iron stores and also help in regulating iron absorption.

## DISCUSSION

Relationship between iron and colorectal cancer has been supported by multiple laboratory and clinical investigations. Increased body iron affords proliferative advantage and tumor growth (Shamsuddin and Ullah 1989). This becomes more relevant for colon, as the unabsorbed iron remains in the colon lumen and interacts with colon epithelial cells. It has been shown previously that IP<sub>6</sub> given in water reduces cell proliferation and suppresses carcinogenesis *in vitro* and *in vivo* (Shamsuddin and Ullah 1989). Iron overloading enhances tumor growth in AOM-treated animals, but the effect of exogenous phytate which is capable of chelating, insolubilizing iron and reducing iron bioavailability was not assessed, with an emphasis on the role of iron status. To explore whether dietary administration of phytate affects AOM-induced colon tumorigenesis, F344 rats were fed 0, 1 or 2% phytate. 1% phytate level used in the present study accurately represents the likely intake of phytic acid in vegetarian diet, while 2% phytate is achievable in a vegetarian diet supplemented with 30 g of phytate-rich wheat bran. These concentrations were chosen to evaluate if there is any dose-dependent effect of phytate as chemoprotective agent in a rat colon cancer experimental model. None of the rats in the control group (without AOM administration) developed tumors, when autopsied after 36 weeks of treatment. At the termination of the study, the AOM-alone treated rats, 1% and 2% phytate-treated rats during post-initiation period had a tumor incidence of 100%, 67% and 33%, respectively. With two different doses of dietary phytate used in the study, the percentage of inhibition of tumor formation was more in animals given 2% phytate than 1% phytate post-initiation. The influence of dietary phytate in the genesis of colon cancer has received impetus from various animal models. Nielson et al. (Nielson et al. 1987) documented that dietary phytic acid supplementation for 2 months could suppress the mitotic index in the descending colon in control rats. It is clear that the early changes in the colonic epithelium induced by azoxymethane lead up to neoplastic cells, with ACF as preneoplastic lesions appearing before 20 weeks. The colonic epithelial cells with ACF are further triggered into active proliferative phase by iron, increasing the risk of colon adenomas. Phytate counteracted the increased proliferation in AOM-administered animals, through increased

apoptosis. Although normal levels of dietary iron were incorporated in the diet in phytate supplemented groups, azoxymethane-induced colonic tumor promotion and progression could be effectively suppressed due to lowered bioavailability of iron. Rats treated with azoxymethane and provided either 1 or 2% phytic acid in drinking water also showed decrease in colon tumor frequency, to the extent of 34.8% and 76.4%, respectively (Shamsuddin and Ullah 1989). Phytate, thus appears to reduce the bioavailable iron and thereby reduce the colonic cell proliferation, although other iron chelators have been shown to alter expression of various genes relevant to cell cycle and tumor suppression (Yu et al. 2007). It should be emphasized that phytate supplementation, but not AOM administration alone (induction) was associated with symptoms of iron deficiency and anemia. Low hepcidin levels in phytate supplemented rats suggest a tendency for promoting dietary iron absorption and mobilization of iron from intracellular stores. These results clearly suggest that ACF progression into tumors is suppressed by phytate, which coincided with overall decrease in expression of various marker proteins in colon tissues.

Mutations in the Wnt pathway account for nearly 90% of the colon cancer tumors (Giles et al. 2003). In addition to excessive  $\beta$ -catenin expression, COX-2 overexpression also contributes to the development of colon cancer, as reported by Spychalski et al. (Spychalski et al. 2007). Our study showed enhanced expression of  $\beta$ -catenin and COX-2 in AOM-alone administered animals and the ability of phytate to decrease the expression of these marker proteins.  $\beta$ -catenin is a key regulator of the Wnt signaling pathway.  $\beta$ -catenin normally localizes to the cytoplasm and upon phosphorylation is degraded by the ubiquitin-proteasome system. In tumors,  $\beta$ -catenin is dephosphorylated resulting in cytoplasmic accumulation as well as translocation to the nucleus, to activate the transcription of Wnt target genes (Verheyen and Gottardi 2010). It was evident that nuclear  $\beta$ -catenin content increased with AOM but decreased significantly upon phytate-supplementation. The overall accumulation of  $\beta$ -catenin in the nucleus correlated with increased tumor burden and multiplicity. This finding indicated that phytate has the potential to reduce  $\beta$ -catenin expression. Thus, it can be speculated that phytate might represent a natural

chemopreventive agent that acts via the Wnt pathway, involving  $\beta$ -catenin. Weak COX-2 expression in normal colonic mucosa and increased expression in AOM-induced animals was consistent with many other studies (Rao et al. 1999). This finding was further supported by Suzuki et al. (Suzuki et al. 2007), who found high levels of COX-2 in AOM-induced colonic mucosa. Previous studies have also shown that COX-2 can be upregulated *via* nuclear  $\beta$ -catenin accumulation, which might be associated with transcriptional regulation by the Wnt signaling pathway (Araki et al. 2003). Reduction in the intensities for COX-2 was observed in colon tumors from rats treated with phytate diet, demonstrating the anti-inflammatory property of phytate. This finding was consistent with the results obtained previously (Roschek et al. 2009), which showed that stabilized rice bran extracts containing phytate inhibited COX-2 activity. These results suggested that dietary administration of phytic acid reduced colon tumor multiplicity *via* an anti-inflammatory mechanism involving  $\beta$ -catenin accumulation and COX-2 expression. The growth rate of tumors can be determined using the rate of proliferation and death of tumor cells (Qiao et al. 1997). One of the most reliable indicators to examine colorectal cell proliferation is the evaluation of proliferating cell nuclear antigen (Biasco et al. 1994). PCNA is an auxiliary protein of DNA polymerase- $\delta$  and high levels of its expression correlate with cell proliferation, suggesting that PCNA is an excellent marker of cellular proliferation (Shimazaki et al. 2005). Our data revealed that PCNA expression was barely detectable in the control group but was significantly higher in colorectal tumor tissue from the AOM-alone group ( $P < 0.05$ ), compared to 1% and 2% phytate. Reduction in cell proliferation observed is one of the modes of action by which phytate is thought to exhibit its chemopreventive efficacy and a significant reduction in cell proliferation was observed with both 1% and 2% phytate ( $P < 0.05$ ), which further support the decreased incidence of hyperplastic foci and tumors. A reduction in tumor incidence is generally correlated to a decrease in cellular proliferation and/or increase in apoptosis (Barnes et al. 1996). As shown in Table 1, histological evaluation using H&E staining indicated that the colon tumor incidence and tumor multiplicity were much higher in the AOM-alone group compared to the phytate supplementation groups. This finding indicated that dietary phytate not only inhibited cell proliferation,

but induced apoptosis in colon tumor tissue. Thus, this may represent an appropriate approach to suppress the promotion and progression of carcinogenesis *via* the induction of apoptosis by dietary chemopreventive agents 1, 2-dimethylhydrazine (DMH) and azoxymethane are commonly used carcinogens that initiate colorectal tumorigenesis in rodent models. Chronic treatment with these carcinogens up to 20-weeks will lead to a tumor response with a 100% incidence with multiple tumors (Rosenberg et al. 2009). In mice fed iron-rich diets, DMH-induced colon tumors were robustly increased with respect to tumor size and multiplicity (Siegers et al. 1988). Consistent with this data, iron enriched diets increased colon tumorigenesis in genetic models of colon cancer (Radulescu et al. 2012). Two separate studies using genetic mouse models of CRC demonstrated that mice on low-iron diet had decreased colon tumors compared to mice on an iron-rich diet (Radulescu et al. 2012). A robust decrease in tumor cell proliferation was noted in both studies following low iron treatment. In a similar manner, feeding different levels of phytate resulted in different levels of iron depletion and resulted in decreased incidence of tumors in the present study. Higher level of transferrin saturation resulted in subsequent development of cancer (Stevens et al. 1994), although other measures of iron status were not determined. Though serum concentrations of ferritin are not tumor-specific, it is also considered a useful prognostic indicator (Wang et al. 2010). Phytate supplementation in the present study clearly showed lowered serum hepcidin levels, indicating impaired iron status and development of iron deficiency (Pasricha et al. 2011). Lowered liver iron and circulating ferritin concentration, along with hepcidin also depict the depleted iron stores (Goddard et al. 2011). These changes in iron status observed with phytate supplementation can be attributed to the lowered bioavailability of iron, due to in solubilization of dietary iron by phytate. Similar alterations that result in increased iron acquisition and decreased iron export by the tumor are also observed for other tumors such as breast (Jiang et al. 2010). These data demonstrate that local iron requirements are increased in tumors compared to normal tissues, and the increase in intra-tumoral iron may be critical in tumor growth. It appears that the iron homeostasis was maintained in AOM-administered animals despite increased iron requirement in the colon tissue.

However, phytate supplementation lowered the iron bioavailability and altered the iron homeostasis, thereby suppressing the colon cancer.

## CONCLUSION

Thus, the present study unequivocally demonstrates that dietary phytate supplementation in AOM-administered animals effectively decreased the incidence of aberrant crypt foci and tumors, through lowered iron status and modulated the cell proliferation, apoptotic and inflammatory changes.

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## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

PVSP, SB, and VLB participated in the design of the study. PVSP and SB carried out the experiments. PVSP performed acquisition of data. PVSP and SB contributed to the analysis and interpretation of data. SB and VLB drafted the manuscript. All authors read and approved the final version of the manuscript.

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