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Co-treatment with Sulforaphane-zein microparticles enhance the chemopreventive potential of Zinc in a 1, 2-Dimethylhydrazine induced colon carcinogenesis rat model

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Abstract

Decrease in plasma and tissue zinc is associated with the development of pre-neoplastic lesions, correlating well with the progression to carcinoma in the colon. There is also a decrease in antioxidant proteins like the zinc dependent Metallothionein (MT) and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) because of the overwhelming oxidative stress. Sulforaphane (SFN) increases cellular antioxidant capacity by releasing Nrf2 from its inhibitory complex and prevents oxidative damage induced colon carcinogenesis. Administration of zinc also induces synthesis of MT directly and indirectly via its action on Nrf2. Because zinc and SFN both act on MT and Nrf2, we evaluated effects of co-administration of zinc and SFN formulated as microparticles with zein in a 1, 2-Dimethylhydrazine (DMH) induced colon carcinogenesis rat model. Groups of rats were sacrificed after the end of four and six weeks and appearance of non-dysplastic, hyperplastic and dysplastic types of aberrant crypts were considered as biomarkers for evaluating the process of carcinogenesis. Colonic tissue levels of MT and Nrf2 were measured along with other proteins and enzymes to evaluate oxidative stress load in the colonic tissue. The co-treatment showed significantly greater reduction in formation of aberrant crypts along with increased induction of cellular antioxidant components compared to single treatments. This investigation supported the hypothesis that co-administration of zinc and SFN leads to an enhanced chemopreventive outcome mainly facilitated by the induction of MT and Nrf2.

Key words: carcinogenesis; aberrant crypt foci; sulforaphane; oxidative stress; chemoprevention

Introduction

A major culprit in development of cancer is an unhealthy diet. By adopting a healthier lifestyle and taking appropriate dietary measures it is possible to prevent 30-40% of cancers.[1] Phytoconstituents and dietary fibre have a lot to do with intestinal transit, the bulk and consistency of stools and along with certain other factors like luminal pH and production of mucus, responsible for changes in intestinal bacterial flora.[2] Active carcinogens produced due to the action of abnormal micro flora on dietary elements if held in a concentrated form in contact with bowel mucosa for prolonged periods due to low fibre content can lead to colorectal cancer and this is one of the reasons why colorectal cancer cases are higher among people in economically developed countries.[3] The mechanistic ways in which poor diets affect health status and aid carcinogenesis have been the topics of research in recent years and in this study we intended to focus on the antioxidant properties of zinc, an essential dietary trace element and its effect on colon carcinogenesis especially in presence of Sulforaphane (SFN) - a second generation antioxidant.

Oxidative stress has been accused of initiating carcinogenesis in most cancers and the role of antioxidants in cancer protection has been reviewed critically.[4] Due to long term exposure to carcinogen or oxidative stress, the delicate balance between accumulation of free radicals within cells and ability of the cells to recruit antioxidant enzymes gets disturbed leading to initiation of carcinogenesis.[5]

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is considered a master regulator of the cellular anti-oxidant system.[6] Nrf2 is held in the cytoplasm as an inactive protein complex with Kelch like ECH-associated protein 1 (Keap1) and Cullin 3 (Cul3). This complex is susceptible to ubiquitination and proteasomal degradation in the cytoplasm. Under oxidative stress the link between Keap1 and Nrf2 is lost and the free Nrf2 now migrates into the nucleus where it can react with the

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antioxidant response element (ARE) gene triggering the transcription of a host of antioxidant enzymes.[7]

SFN is a naturally occurring phytoconstituent belonging to the isothiocyanate family of compounds and is present in high concentrations in broccoli (*Brassica oleracea*).[8]

It can act by virtue of its Histone deacetylase inhibitory (HDACi) activity either directly on Keap1 by interacting with its sulfhydryl groups and releasing Keap1 or indirectly by activation of the RAS-Raf1-MEK-ERK2 pathway thus causing phosphorylation of Keap1 and liberation of Nrf2.[9]

Metallothioneins (MTs) are a group of cysteine rich, low molecular weight intracellular proteins and expression of MT has been related to activation of cellular antioxidant systems.[10] MT expression is critically dependent on Metal Responsive Element (MRE) promoter activation which is activated by MRE binding transcription factor-1 (MTF-1). MRE binding protein (MREBP) binds to MRE and keeps it in an inactive form. Metallothionein transcription inhibitor (MTTI) associates with MTF-1 and prevents its interaction with MRE.[11] Free zinc causes dissociation of the MTTI/MTF-1 complex and releases MTF-1 for interaction with MRE. Zinc also liberates MRE from the repressive action of MREBP and thus promotes MT transcription.[12]

Zinc is an essential element that needs to be present in an optimum amount and this is maintained by zinc transporters whose expression are altered in many cancers.[13] It is known that zinc status in colon cancer patients deteriorates and the decline in plasma zinc promotes carcinogenesis while the opposite may be beneficial.[14-16]

Apart from its action on MT, zinc also increases basal Glutamate-cysteine ligase catalytic subunit (GCLC) synthesis by increasing translocation of Nrf2 to the nucleus.[17] Further, SFN has been shown to possess anti-proliferative effects in HepG2 cells by inducing expression of MT.[18]

Over expression of MT and Nrf2 in later stages of carcinogenesis may allow cancer cells to evade oxidative stress damage due to chemotherapy and thus become resistant to treatment.[19, 20] However, their early induction is expected to prevent oxidative stress induced DNA mutations and act as cancer protectives.[21]

Aberrant crypt formation is a valid and well documented histopathological change that occurs during the early stages of colon carcinogenesis.[22] In this study, 1, 2-Dimethylhydrazine (DMH) induced appearance of aberrant crypts in the rat colon was considered as short term (four and six weeks) endpoint as they correlate well with development of tumors at around 30 weeks.[23]

The objective of the present study was to evaluate the chemopreventive outcome upon co-administration of zinc and SFN in DMH treated rats in comparison with single treatment with either zinc or SFN.

Experimental methods

1, 2-Dimethylhydrazine dihydrochloride and Sulforaphane standard were purchased from Sigma Aldrich, USA and Zinc sulphate was purchased from SD Fine Chemicals Ltd., Mumbai, India. The microparticles of SFN with Zein (a prolamin protein from maize) were formulated at DBT-ICT-Centre for Energy Biosciences (CEB), Institute of Chemical Technology, Mumbai, India, and further studies were carried out at Bombay College of Pharmacy, Kalina, Mumbai, India.

Preparation and characterization of SFN - Zein microparticles

SFN was obtained by crushing broccoli florets (10 kg) with water and maintaining pH 6.5 ± 0.5 for a period of 2 hours with slow stirring. At these conditions, Glucoraphanin, a precursor of SFN gets extracted and acted upon by myrosinase to form SFN. The extraction mixture was centrifuged at 10,000 rpm to recover 7.4 litre of supernatant. SFN from supernatant was then captured on 200 mL of ODS silica and eluted with 500 mL of 88% v/v isopropyl alcohol. 29 g of Zein was added to the eluate containing 724 mg of SFN and then the solvent was removed from this mixture by vacuum of 9 mbar at 40^0C yielding SFN - Zein microparticles - a solid preparation of SFN which otherwise was an oily liquid.

High Performance Liquid Chromatography (HPLC) analysis

SFN analysis was carried out on HPLC (Agilent Technologies 1200 series) using PuroSphere STAR C18 column (250 mm x 4.6 mm, 5 µm particles) at 36^0C, using a flow rate of 0.6 ml/min and detection at 202 nm with a mobile phase of Water: Acetonitrile (70:30).

Scanning Electron Microscopy (SEM)

Analytical scanning electron microscope (SEM, JSM-6380 LA, JEOL Ltd., Tokyo, Japan) was used to characterize and study morphology of the SFN loaded zein formulation. Surface features and shape of the microparticles were determined from SEM images. Sample preparation for SEM was carried out by covering the formulation / concentrate with conductive platinum metal coating using auto fine coater (JFC-1600, JEOL Ltd., Tokyo, Japan).

Electrospray ionization–Triple quadrupole mass spectrometry analysis

HPLC fractions of SFN detected in the microparticle formulation were collected and analyzed by using electrospray ionization– triple quadrupole MS (ESI-Triplequad-MS/MS). An Agilent 1200 HPLC system consisting of binary pump and autosampler was used. The Triple quad mass spectrometer was equipped with a turbo ion spray source (Agilent 6410 model). Positive ion mode was used for the analysis of the SFN. The mobile phase was 0.1% formic acid in water with a linear gradient of acetonitrile over 2 min. The flow rate was 0.5 ml/min and injection volume was 2 µl. Drying gas flow rate was 8 L/min, nebulizer gas (N_2) pressure 45 psi and drying gas (N_2) temperature 350°C. Full-scan LC-MS spectra were obtained by scanning from m/z 100 to 200. Agilent technologies LC/MS Triple Quad equipped with Mass Hunter Workstation B.03.01Version software was used for detection of SFN. The MS/MS detection was achieved using positive ion mode with an m/z transition of 177.3 for SFN.

Animal study

Six week old female Sprague-Dawley rats in the weight range of 180 to 200 g were obtained from Piramal Healthcare Ltd., Mumbai, India and were housed in a temperature regulated environment (24-28°C) with a 12-h light and dark cycle. Throughout the study they were maintained on a pelletized diet purchased from Pranav Agro Industries Ltd., Sangli, India.

Induction of carcinogenesis

After ten days of acclimatization, rats were randomized into two groups, Group 1 - Vehicle (saline) control and Group 2 – DMH treated, in which rats were administered DMH (30 mg/kg, i.p.) twice weekly for three weeks and were sacrificed at various time points to observe histopathological tissue level changes in a graded manner over a period of 2 to 16 weeks (Figure 1).

Evaluation of chemopreventive effects of zinc and SFN

For this study, rats were randomized into the various treatment groups (n=10/group) as indicated in Table 1.

Rats in the groups 3 and 5 were allowed access to zinc in the form of Zinc sulphate (227 mg/L) via drinking water *ad libitum* and the rats in groups 1 and 4 were provided plain drinking water *ad libitum*. SFN-zein formulation equivalent to a dose of 10 µmole/kg of SFN was suspended in soy oil and orally administered to rats in groups 4 and 5.

Five rats from each group were sacrificed at the end of 4 weeks and the remaining at the end of 6 weeks. The colons of the rats were isolated for further analysis. The protocols used in the study were approved by the Institutional Animal Ethics Committee (IAEC) of Bombay College of Pharmacy, Kalina, Mumbai, India.

Water consumption and body weight measurements

Volumes of water consumed over intervals of 48 hours were measured and averaged out for the number of rats in the cage. Body weights were noted weekly using animal weighing balance (Rudra Systems-121P, India).

Isolation of colon tissue

After humanely sacrificing the rat**s** using CO_2 chamber, colons were isolated and were perfused clean with cold phosphate buffered saline (PBS), pH 7.4. For histopathology the colons were slit open longitudinally and placed individually in 10% Neutral buffered formalin (NBF). For biochemical analyses colon tissues, free of luminal contents, were homogenized (REMI Motors/RQ-127A) with chilled PBS (1:5), centrifuged at 7000 rpm for 10 minutes and the supernatants were stored at -70°C until analysis.

Measurement of MT and Nrf2

MT and Nrf2 protein levels in colonic tissue homogenates were measured by ELISA kits based on Biotin Double Antibody Sandwich Technology (Shanghai Yehua Biological Technology Co. Ltd., China).

Measurement of Catalase activity (CAT)

The method described by Asru K. Sinha (1972)[24] was adopted with slight modifications. The method is based on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured by UV-Vis spectrophotometry at 570-610 nm. Briefly, dichromate/acetic acid reagent was prepared by mixing 5% $K_2Cr_2O_7$ with glacial acetic acid (1:3) and 2 ml of this was added to each labelled test tube. 0, 10, 20, 30, 40 and 50 µl of a 2.02 M H_2O_2 stock was added to each tube and used to prepare a calibration curve. All test tubes were made up to 3 ml with 0.01 M PBS (pH 7.4). After heating on a water bath at 72°C for 10 minutes and cooling to room temperature, again the volume was made to 3 ml with PBS. Absorbance of the supernatant was measured at 570 nm after centrifugation at 3000 rpm for 10 minutes. For the sample, 100 µl of tissue homogenate was added in the tube after addition of 30 µl of H_2O_2. From the calibration curve, the amount of H_2O_2 remaining was calculated and correspondingly amount of H_2O_2 decomposed was determined by material balance. Catalase activity was expressed as mM of H_2O_2 decomposed per mg of protein.

Measurement of Reduced Glutathione (GSH)

The GSH content in the colon homogenate was measured by the method of Jollow *et al.,* (1974)[25] in which 0.5 ml of tissue homogenate was mixed with 0.5 ml of 4% Sulphosalicylic acid and incubated at 4°C for one hour. Centrifugation was done at 3000 rpm at 4°C for 15 minutes. To 27 µl of supernatant, 145 µl of 0.1 M PBS (pH 7.4) and 27 µl of 10 mM 5, 5'-Dithiobis-(2-nitrobenzoic acid) or Ellman's reagent were added. Absorbance was read immediately at 412 nm. GSH levels were expressed as micromoles of DTNB conjugate formed per mg of protein.

Measurement of Superoxide dismutase activity (SOD)

SOD activity was measured as per the method of Misra and Fridovich (1972)[26] with a few alterations. In this method, 2 ml of 0.05 M carbonate buffer (pH 10.7), 0.5 ml of 0.1 mM EDTA and 50 µl of tissue homogenate were taken in a glass tube. The reaction was initiated by adding 0.5 ml of 3 x 10^{-4} M Epinephrine. The absorbance was measured immediately at 480 nm for 3 minutes against blank in which no tissue homogenate but 2.5 ml of carbonate buffer was used. Values were reported as units of SOD per mg protein.

Measurement of Lipid Peroxidation (LPO)

Extent of lipid peroxidation was measured by the Thiobarbituric acid reactive substances (TBARS) method described by Buege and Aust (1978)[27] with certain alterations. Briefly, 0.1 ml of 150 mM Tris-HCl buffer, 0.1 ml of 1 mM

$FeSO_4$, 0.1 ml of 1.5 mM Ascorbic acid and 0.1 ml of tissue homogenate were taken in a glass tube. The volume was made up to 1 ml with water and the contents were incubated at 37°C for 15 minutes. After cooling to room temperature, 1 ml of 10% Trichloroacetic acid (TCA) and 2 ml of 0.375% w/w Thiobarbituric acid (TBA) were added and sample was incubated at 37°C for 15 minutes. After cooling and centrifugation at 3000 rpm for 10 minutes, absorbance of the supernatant was read at 532 nm. Extent of lipid peroxidation was expressed as µmoles of Malondialdehyde (MDA) formed per mg protein.

Estimation of total protein

Total protein in the colon tissue homogenates were measured by the method of Hartree (1972)[28] using Bovine Serum Albumin (BSA) as a standard.

Histopathology

5 µm thick sections of colon tissues were cut from paraffin wax blocks using a microtome (Thermo Scientific-HM355S), fixed on clean glass slides and stained with Haematoxylin and Eosin (H&E). The stained slides were observed for appearance of aberrant crypts under a microscope (Motic DMBA310) at a magnification of 10x and 40x.

Identification of non-dysplastic, hyperplastic and dysplastic aberrant crypts

ACF were classified into foci showing no dysplasia (non-dysplastic), hyperplasia (hyperplastic) or foci showing dysplasia (dysplastic) and were considered as short term events occurring before tumor formation as they have been well documented.[29, 30] ACF with no significant morphological changes in the mucosa but having an enlarged lumen compared to normal crypts were considered as non-dysplastic ACF.[31] Hyperplastic ACF were those showing an increased proliferative zone and a serrated lumen lined by hyperplastic epithelium.[32] Dysplastic ACF, having the highest probability to turn cancerous[33], were characterized as having a dilated but thickened and closing lumen with loss of nuclear polarity.[34] Quantitative analysis of the crypts was done using Photoshop® CC2014 (Adobe systems, Inc., San Jose, CA) by counting the number of crypts/mm^2 of colon tissue.[35]

Statistical analysis

All data were analyzed by One Way Analysis of Variance (ANOVA) and significant differences were tested using Tukey's *post hoc* test. The results were considered significant when $p<0.05$ (95% confidence interval). All statistical analyses were done using Prism Version 6 (GraphPad Software Inc.), USA.

Results and Discussion

SFN-Zein microparticles

In this study, SFN was formulated as microparticles with zein yielding a solid preparation compared to the otherwise oily form of SFN.

Analysis of SFN-loading in the microparticles was done using HPLC which was found to be 2.425 % w/w (Supplementary Figure 1).

The SEM images of formulation is presented in Supplementary Figure 2. It can be observed that in the zein based SFN formulation, the surfaces are irregular and characterized with presence of prominent holes. It has been reasoned out that the holes seem to be the footprint of entrapped air bubbles formed due to evaporation of solvent molecules during preparation of the hydrophobic protein based formulations. In the current research SFN loaded zein microparticles in the size range of 5-15 µm were prepared by evaporation method which was found to be superior to phase separation method in terms of encapsulation efficiency and microparticle yield (unpublished data).

LC/MS chromatographs of the standard SFN and formulated SFN microparticles are shown in Supplementary Figures 3A and 3B respectively. These result shows SFN product ion peak $(M+H)^+$ (m/z177.70). The fragment ion peaks obtained m/z 114 $(M\text{-}CH_3OS)^+$ and m/z 118.90 $(M\text{-}NCS)^+$ which were not the isotope of abundant molecular ion (m/z 177.70). The LC/MS ESI mode spectra of purified and standard samples were identical. The results obtained in this study were similar to a previous report.[36]

Induction of colon carcinogenesis

DMH is a potent colon carcinogen used to induce colon cancer in rodents similar to sporadic colon cancer in humans.[37] It acts by increasing oxidative stress in colonic tissue by increasing the levels of reactive oxygen species (ROS).[38, 39] After conversion into the active form Methylazoxymethanol (MAM), it interacts with the DNA and induces oxidative stress that is fatal to the cells.[40] This reduces the tissue's capacity of producing antioxidant enzymes to combat the rising oxidative stress. The accompanying DNA damage may induce DNA mutations leading to carcinogenesis.[41] Colon specificity and well-studied dose dependency make DMH an ideal inducer of colon carcinogenesis.

The histopathology results obtained in our study by the end of the 16^{th} week with the initiation of carcinogenesis between 4^{th} and 6^{th} weeks were in agreement with reports of appearance of aberrant crypts and precancerous lesions (Figure 2).

Alteration in body weights and water consumption pattern

In order to see if the treatments had any effect on body weight, weekly body weight measurements were taken and analysed (Figure 3A).

Zinc alone did not cause any significant weight gain. A significant weight gain was found in the groups treated with SFN, either alone or along with zinc, compared to the positive control. Treatment with SFN has been shown to be beneficial to reduce cholesterol and prevent body weight gain in obese animals.[42] In our study however, we found that SFN facilitates body weight gain. This may be due to an improved overall health condition of the rats upon treatment with SFN.

When water consumption pattern for each 48 hour interval was analysed (Figure 3B), we found a significant reduction in volume of drinking water consumed per rat in groups treated with zinc either alone (40.81±2.84 ml) or when SFN was also co- administered (42.27±3.09 ml) compared to the negative control group (53.67±2.18 ml).

We also found that in zinc fed groups, there was a reduction in drinking water consumption. This could be due to the change in taste of the drinking water when Zinc sulphate was dissolved in it. The average zinc consumption per rat over a 48 hour interval was calculated to be 9.26±0.64 mg and 9.59±0.70 mg for groups 3 and 5 respectively.

Colonic tissue levels of MT and Nrf2

One of the major role-players in the cellular antioxidant system is MT which is induced in the presence of oxidative stress.[43] However, analysis of MT levels in the colon tissue homogenates (Figure 4A) revealed that DMH treatment decreased MT levels significantly (12.79±0.81 ng/g after 4 weeks and 11.33±1.52 ng/g after 6 weeks) compared to the negative control (42.10±1.07 ng/g after 4 weeks and 37.39±3.49 ng/g after 6 weeks). This is in contrast to other studies that report a higher MT level during oxidative stress.[44, 45] Only in the group treated with both Zinc and SFN, a significantly increased MT (33.43±2.19 ng/g after 4 weeks and 43.17±3.43 ng/g after 6 weeks) was seen which was comparable to the negative control group. Units of tissue MT levels are ng/g tissue.

As mentioned earlier, Nrf2 is responsible for induction of a number of genes required for maintaining cellular antioxidant status including GCLC.[17] In resonance with the findings on MT, a similar trend was observed with Nrf2 (Figure 4B) where a significant decrease in the positive control group (32.16±2.78 ng/g after 4 weeks and 26.60±1.73 ng/g after 6 weeks) was seen compared to the negative control (59.18±2.55 ng/g after 4 weeks and 64.17±0.21 ng/g after 6 weeks). Only in the group treated with both Zinc and SFN, a significantly increased Nrf2 (60.98±2.30 ng/g after 4 weeks and 70.56±1.31 ng/g after 6 weeks) was seen. Units of tissue Nrf2 levels are ng/g tissue. Statistical analysis clearly demonstrated that co-administration of SFN with Zinc had significantly enhanced responses compared to single treatment. We propose that the MT and Nrf2 levels were lower in the positive control due to their excessive utilization when the tissue is initially exposed to high oxidative stress as reported.[46] The cellular antioxidant system could have been overwhelmed by the oxidative stress, leading to a drastic fall in MT and Nrf2 in the positive control group.

Evaluation of antioxidant markers in colonic tissue homogenates

Nrf2 being a master regulator of various antioxidant enzymes, we measured the activities of enzymes like Catalase and Superoxide dismutase as well as levels of Reduced Glutathione and the extents of Lipid Peroxidation to estimate the load of oxidative stress on the rat colon.

Catalase activity

Catalase is an enzyme that catalyses the decomposition of hydrogen peroxide to water and oxygen.[47]

The enhancement in CAT activity was not significant when treated with either Zinc (8.99±0.07 mM after 4 weeks and 9.10±0.65 mM after 6 weeks) or SFN (10.98±0.51 mM after 4 weeks and 10.80±0.49 mM after 6 weeks). However, the increase was significant (12.39±0.62 mM after 4 weeks and 14.36±1.02 mM after 6 weeks) when rats were co-treated with both the Zinc and SFN (Figure 5A). Tissue CAT activities are expressed as mM H_2O_2decomposed per mg protein.

Reduced GSH

This hydrophilic tripeptide composed of glutamate, cysteine and glycine and present predominantly in the cytosol, prevents the build-up of potentially damaging free radicals and thus functioning as a very important antioxidant protein.[48] The level of reduced GSH form is about 100-fold times higher than its oxidized GSSG form.[49] There is a decrease in the intracellular GSH pool during colon carcinogenesis and gradual decline in the antioxidant status leads to progression to colon cancer.[50]

Treatment with Zinc alone (0.35±0.01 µM after 4 weeks and 0.58±0.02 µM after 6 weeks) and SFN alone (0.44±0.01 µM after 4 weeks and 0.51±0.06 µM after 6 weeks) increased GSH levels significantly compared to the positive control but co-treatment with both agents simultaneously showed greater GSH levels (0.61±0.01 µM after 4 weeks and 0.75±0.01 µM after 6 weeks) (Figure 5B). Tissue GSH levels are expressed as µmoles of DTNB conjugate formed per mg protein.

Superoxide Dismutase activity

The expression of SOD has generally been found to be lower in cancer tissues in relation to their normal counterparts.[51]

Treatment with either Zinc (15.31±0.95 units after 4 weeks and 14.47±0.14 units after 6 weeks) or SFN (12.98±0.76 units after 4 weeks and 15.55±0.53 units after 6 weeks) or co-treatment (19.11±1.11 units after 4 weeks and 22.64±1.33 units after 6 weeks), all had a positive effect on SOD activity. However, co-treatment was significantly better compared to single treatments only after 6 weeks (Figure 5C). Tissue SOD activities are expressed as units per mg protein.

Lipid peroxidation

Studies have shown that the reactive aldehydes like 4-hydroxy-2-nonenal (HNE), malondialdehyde, acrolein, and crotonaldehyde, formed as a result of LPO, react with DNA bases to form promutagenic exocyclic DNA adducts contributing to carcinogenesis.[52] The extent of lipid peroxidation being a direct measure of DMH induced oxidative stress was reduced significantly in all treatment groups. When comparing the co-treatment group (4.21±0.13 µmoles after 4 weeks and 4.95±0.03 µmoles after 6 weeks) with the groups that were treated with either Zinc (8.98±0.70 µmoles after 4

weeks and 7.44±0.28 µmoles after 6 weeks) or SFN (5.99±0.11 µmoles after 4 weeks and 5.89±0.10 µmoles after 6 weeks), the reduction in LPO was found to be superior (Figure 5D). Tissue LPO levels are expressed as µmoles of MDA formed per mg protein.

It has been shown that SFN selectively induces apoptosis in cancer cells by increasing ROS and triggering the release of HNE revealing a pro-oxidant mechanism of SFN.[53] HNE has a direct action on Keap1 and disrupts the Keap1-Nrf2 complex.[54]

Proposed mechanism of chemoprevention by Zinc and SFN

In order for the tissue to counter the oxidative stress, an induction of cellular antioxidant defence system is required. Zinc is critical for the functioning of MT and DMH exposure has been shown to lower the level of zinc in tissues including the colon.[16] Keeping this in mind, we administered zinc via drinking water to the rats as previously reported.[46] Zinc interacts with MTF1 after entering into the nucleus and increases MT level in the colonic tissue[11] (Figure 6). MTs have high affinity to cytosolic zinc and bind to them instantaneously, thus maintaining the intracellular zinc in the bound form.[55] Upon exposure to oxidative stress, GSH is first to act upon and convert the free radicals by donating its hydrogen and itself getting oxidized to the Glutathione-S-S-Glutathione (GSSG) form in presence of the enzyme Glutathione peroxidase (GPx).[56] It is the function of Glutathione reductase (GR) to reconvert GSSG to GSH in presence of NADPH and zinc bound MT so that the redox cycle can continue and further ROS can be neutralized. When the balance between generation of ROS and levels of intracellular ROS scavengers is disturbed, GSH is depleted and the ratio of GSH/GSSG falls. In the DMH treated group, a fall in colonic zinc level reduces the intracellular zinc bound MT which correspondingly impedes the recycling of GSSG to GSH.

The entry of externally administered zinc into the colon cells is facilitated by Zrt-and Irt-like proteins (Zip) and efflux transporters like ZnT1, ZnT5 and ZnT7 along with influx transporters maintain intracellular levels of zinc.[57, 58] Via its action on MTF1 and Metal Response Element (MRE) components of the MT promoter, it induces MT expression.[59]

Increased MT will then aid in reconversion of GSSG to GSH. Zinc also increases intracellular GSH mediated by its action on Nrf2.[17, 60] An increased intracellular concentration of free zinc may also lead to bax induced mitochondrial pore formation and release of Cytochrome C which can then trigger the Caspase cascade and apoptosis.[61]

Administration of the HDACi, SFN has been shown to elevate cellular Nrf2 level due to the inhibition of Keap1 as previously reported[7, 21] (Figure 7).

Nrf2 is held in the cytoplasm as an inactive protein complex with Keap1 and Cul3. This complex is susceptible to ubiquitination and proteasomal degradation in the cytoplasm. A delicate balance exists between the levels of free Nrf2 and the complex. Under oxidative stress the link between Keap1 and Nrf2 is lost and the free Nrf2 now migrates into the nucleus where it can react with the Antioxidant Response Element (ARE) gene with the help of certain small molecule Mafs.[62] Activation of ARE triggers transcription of a host of antioxidant enzymes and proteins like Hemoxygenase-1 (HO1)[63], NAD(P)H:quinoneoxidoreductase 1 (NQO1)[64] and Glutamate—cysteine ligase catalytic subunit (GCLC)[65], that is required for the biosynthesis of GSH. Sulforaphane can act by virtue of its HDACi activity either directly on Keap1 by interacting with its sulfhydryl groups and releasing Keap1 or indirectly by activation of the RAS-Raf1-MEK-ERK2 pathway thus causing phosphorylation of Keap1 leading to the liberation of Nrf2.[66]

Apart from its action on Nrf2; chemopreventive potential of SFN is due to its action on Phase I and Phase II enzymes; as an anti-proliferative agent including its action on cell cycle checkpoints, apoptosis, HDAC inhibition of many tumour suppressor genes; facilitates MAPK-ERK activation; promotes NFkB inhibition and affects ROS production.[67] SFN also indirectly affects MT via the Nrf2 pathway[68] which provides a feedback loop for synthesis of MT in the cell. With colonic MT and Nrf2 restored to normal, the capacity to combat DMH induced oxidative stress is reinstated.

Histopathology

Histopathological end points determined were related to the development and quantification of non-dysplastic, dysplastic and hyperplastic aberrant crypts. Histopathology images of the specific types of ACFs observed in our study (Figure 8) were considered as standards for quantification of ACFs and thereby evaluation of the chemopreventive effects.

Microscopy clearly shows that the carcinogen lead to morphological changes in the crypts of the rat colon in agreement with previous reports[69, 70] that were partially prevented upon zinc, SFN and the co- treatment (Figure 9).

Dysplastic aberrations especially have been shown to correlate well with formation of adenocarcinomas and tumours at around 30 weeks.[23] Statistical analysis showed that the co-treatment with Zinc and SFN resulted in a significant reduction in the numbers of aberrant crypts compared to single treatments with either Zinc or SFN (Figure 10).

This supports our hypothesis of enhanced chemopreventive effect by Zinc and SFN co-administration and may be explored further to track its impact on progression of carcinogenesis to a full blown tumor stage.

Conclusion

The results provide evidence to substantiate the hypothesis of this investigation. Co-administration of Sulforaphane simultaneously with Zinc has better treatment outcome in relation to improved colonic tissue antioxidant status and delayed or abated formation of aberrations in colonic crypts as evaluated in this DMH induced colon carcinogenesis model of rats.

Further studies are required to understand the pattern of rise and fall of MT levels during the course of carcinogenesis. Also

in order to establish whether the chemopreventive actions of Zinc and SFN involves synergism or addition, a comprehensive *in vivo* study employing varying doses of both Zinc and SFN is warranted.

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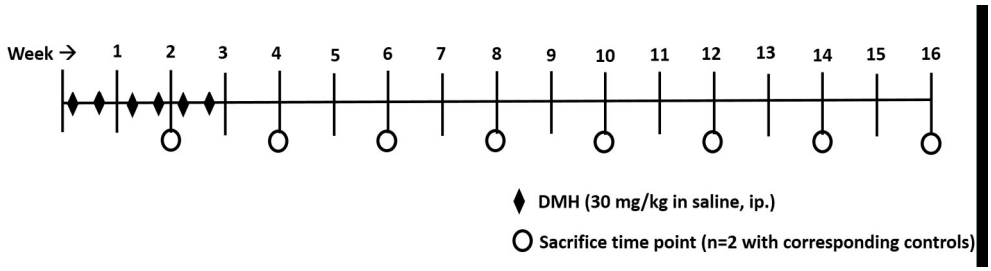

Figure 1 Treatment and sacrifice schedule for the pilot study to evaluate progression of DMH induced carcinogenesis and to establish suitable sacrifice time points to evaluate chemopreventive effects of zinc and SFN
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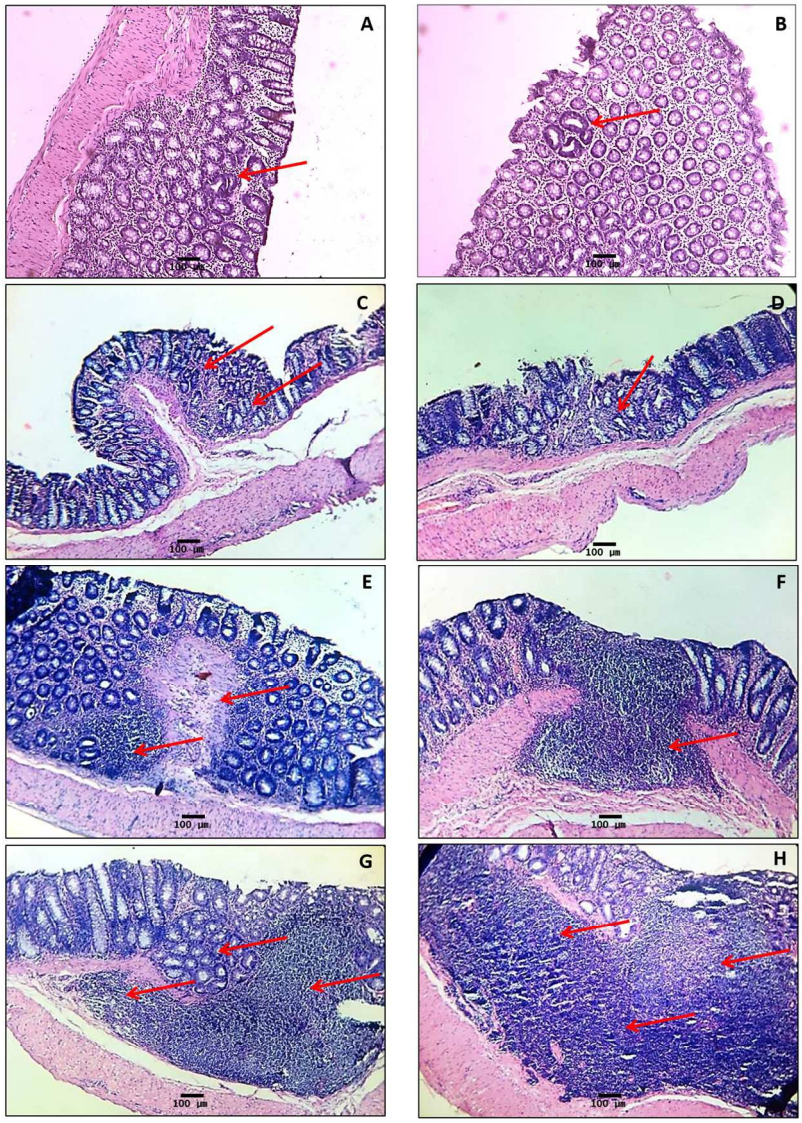


Figure 2 Histopathological images of the Haematoxylin-Eosin (H&E) stained sections of rat colons at 10x magnification at various sacrifice time points. Scale bar=100 µm
A (2 weeks) Crowding of crypts; B (4 weeks) Initiation of aberrations; C (6 weeks) Loss of orderly arrangement of crypts; D (8 weeks) Lymphocytic infiltration and loss of structural morphology of mucosa; E (10 weeks) Full thickness dysplasia in mucosa; F (12 weeks) Full thickness dysplasia in mucosa and lymphoid reaction in mucosa and sub-mucosa; G (14 weeks) Extension of lymphoid reaction into the sub-mucosa; H (16 weeks) Severe lymphoid reaction in entire sub-mucosa
248x343mm (150 x 150 DPI)

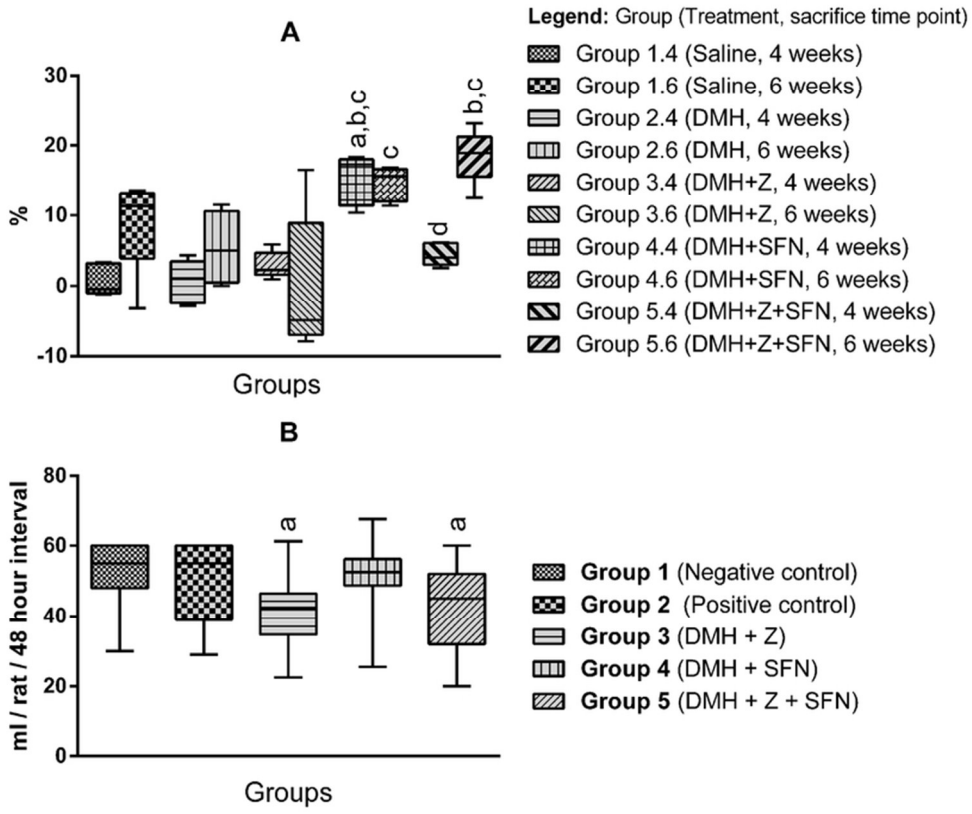


Figure 3 A) Box plot showing Min to Max values for percent change in Body weight from the first week till the week of sacrifice. ap<0.05 compared to group 1, bp<0.05 compared to group 2, cp<0.05 compared to group 3, dp<0.05 compared to group 4 B) Box plot showing Min to Max values for average water consumption per rat in a 48 hour interval. ap<0.05 compared to group 1, bp<0.05 compared to group 2, cp<0.05 compared to group 3, dp<0.05 compared to group 4
35x29mm (600 x 600 DPI)

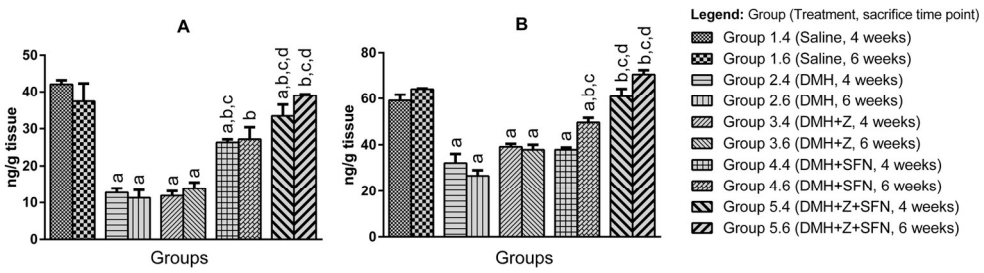


Figure 4 A) Metallothionein levels in colon tissue homogenate B) Nuclear factor (erythroid-derived 2)-like 2 levels in colon tissue homogenate ap<0.05 compared to group 1, bp<0.05 compared to group 2, cp<0.05 compared to group 3, dp<0.05 compared to group 4
76x21mm (600 x 600 DPI)

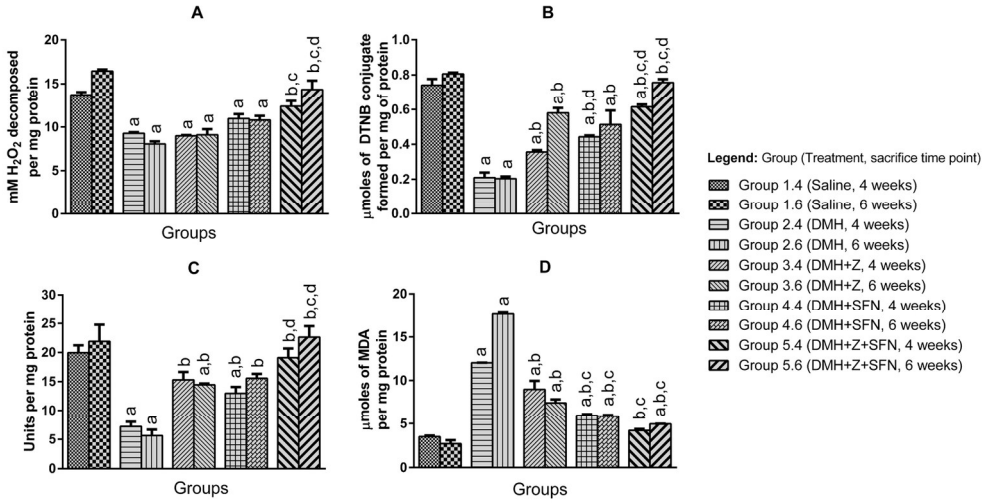


Figure 5 A) Catalase activity B) Level of Reduced Glutathione C) Superoxide Dismutase activity D) Extent of Lipid Peroxidation;
Values are expressed as Mean ± SEM with n=5 for each group. ap<0.05 compared to Group 1, bp<0.05 compared to Group 2, cp<0.05 compared to Group 3, dp<0.05 compared to Group 4
155x82mm (300 x 300 DPI)

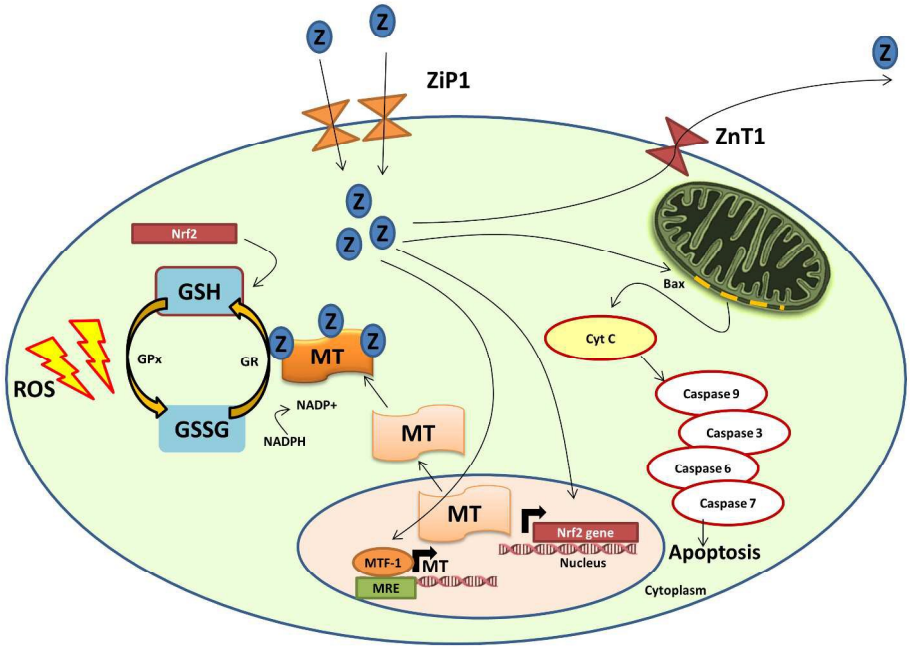


Figure 6 Interplay between intracellular zinc and Metallothionein Abbreviations: Z (Zinc), Zip (Zrt-and Irt-like proteins), ZnT1 (Zinc transporter 1), MT (Metallothionein), GSSG (Glutathione-S-S-Glutathione), ROS (Reactive Oxygen Species), NADPH (Nicotine Adenine Dinucleotide Phosphate Hydrogen), MTF1 (MRE binding transcription factor-1), MRE (Metal Response Element), Nrf2 (Nuclear factor (erythroid-derived 2)-like 2), bax (BCL-2 associated X protein)
254x190mm (300 x 300 DPI)

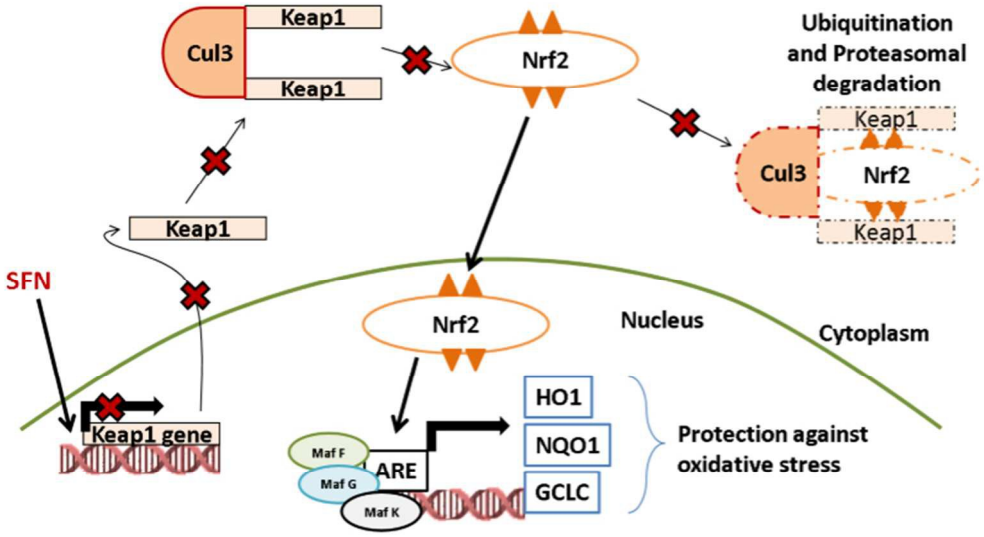


Figure 7 Sulforaphane induced expression of Nrf2
Abbreviations: Nrf2 (Nuclear factor (erythroid-derived 2)-like 2), Keap1 (Kelch-Like ECH-Associated Protein 1), Cul3 (Cullin 3), ARE (Antioxidant Response Element), Maf (Musculoaponeuroticfibrosarcoma)
137x79mm (150 x 150 DPI)

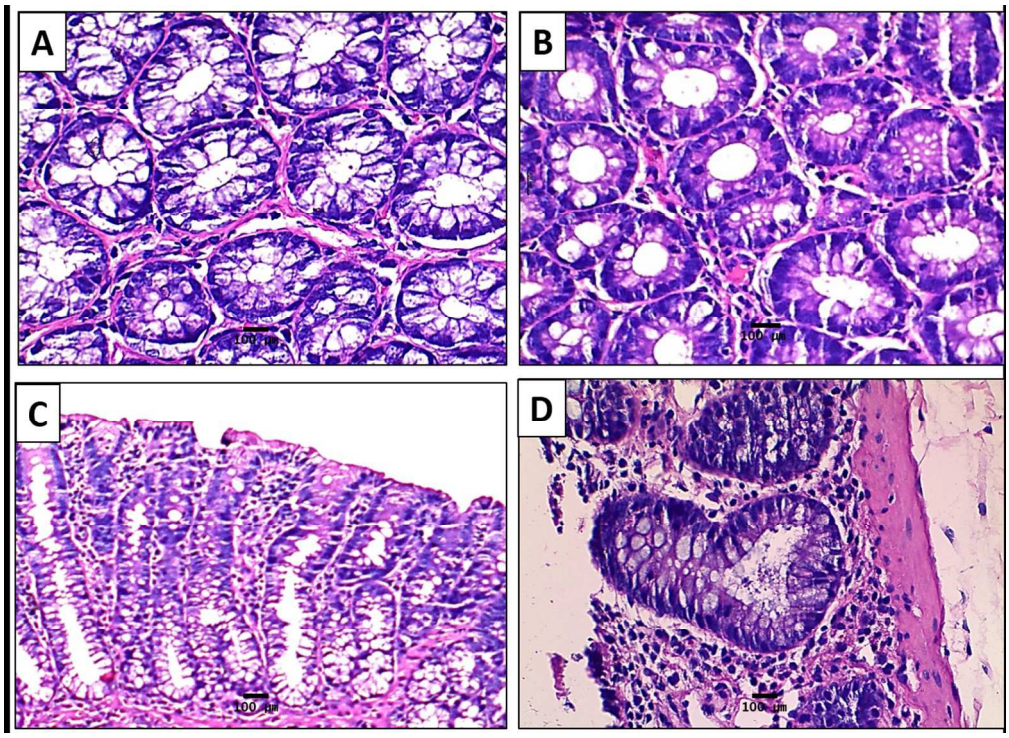


Figure 8 A) Normal crypts, B) Non-dysplastic crypts, C) Hyperplastic crypts and D) Dysplastic crypts; Scale bar = 100 µm
220x160mm (150 x 150 DPI)

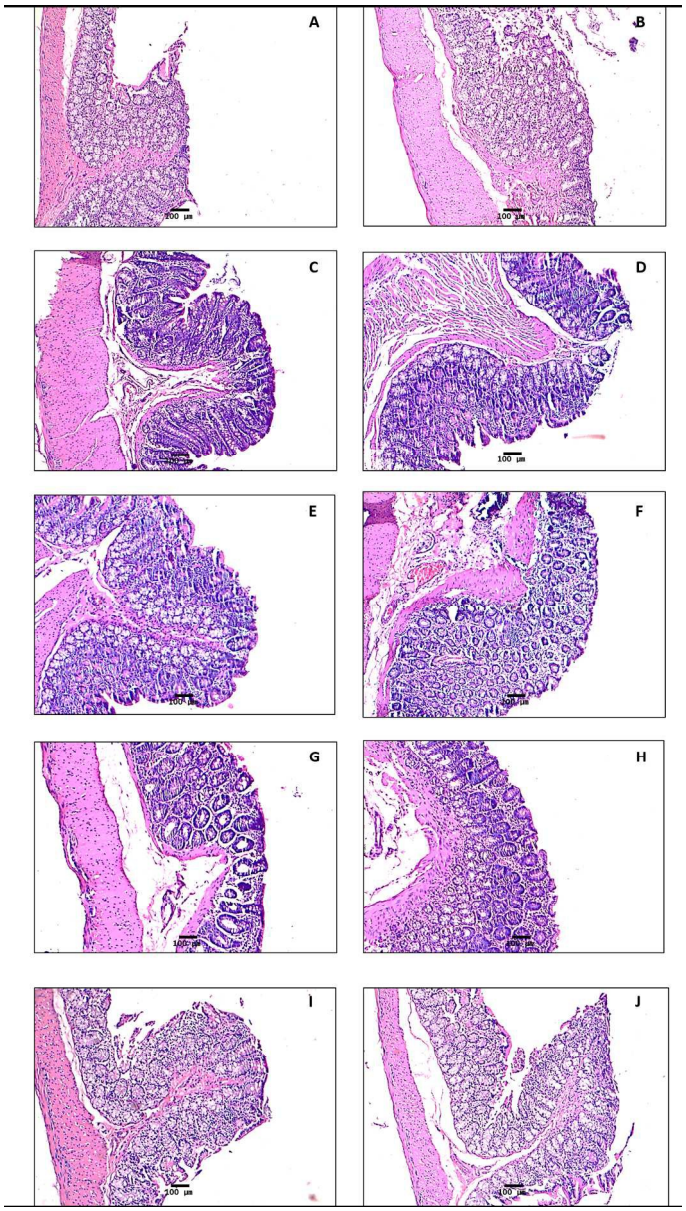


Figure 9 Histopathological images of the Haematoxylin-Eosin (H&E) stained sections of rat colons at 10x magnification; Scale bar = 100 µm
Development of full thickness dysplasia was seen in the group receiving DMH only at 4 and 6 weeks. Treatment with zinc shows sparing of basal crypts from dysplasia which is seen strikingly in superficial crypts at 4 weeks and increased inter-cryptal space and infiltration by lamina propria at 6 weeks. Treatment with SFN reveals no significant abnormalities apart from distortion in arrangement of crypts at 4 weeks. Nuclear atypia and loss of gobletization seen in superficial crypts at 6 weeks. Dual treatment with zinc and SFN shows no severe abnormalities and no detectable dysplasia both at 4 and 6 weeks
243x431mm (150 x 150 DPI)

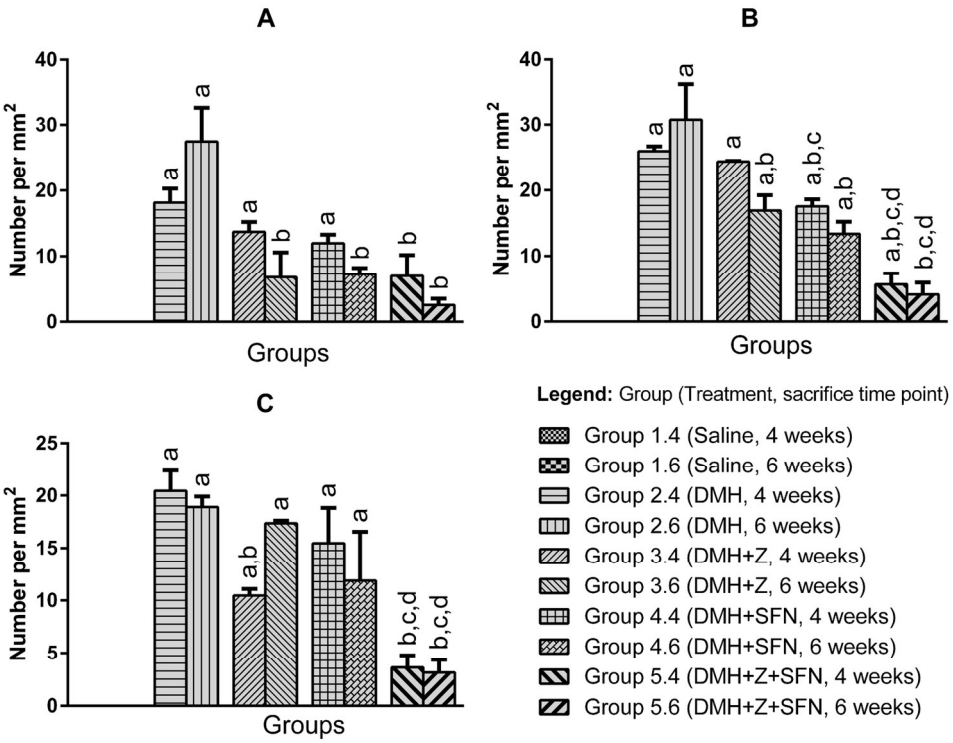
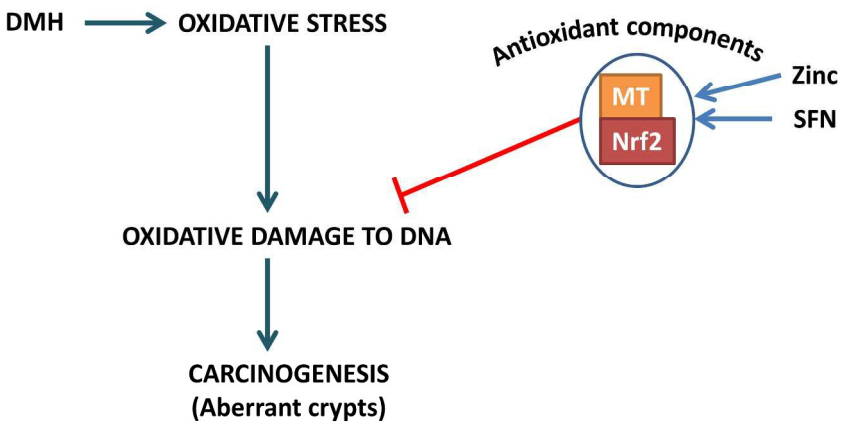


Figure 10 Quantification of A) Non-dysplastic, B) Dysplastic and C) Hyperplastic aberrant crypts in rat colon Values are expressed as Mean ± SEM with n=5 for each group. ap<0.05 compared to Group 1, bp<0.05 compared to Group 2, cp<0.05 compared to Group 3, dp<0.05 compared to Group 4
148x114mm (300 x 300 DPI)

Group	Treatment	Treatment schedule
1	Saline, ip.	Twice weekly for three weeks
2	DMH (30 mg/kg), ip.	Twice weekly for three weeks
3	DMH (30 mg/kg in saline), ip.;	Twice weekly for three weeks
	Zinc sulphate (227 mg/L) in drinking water	*ad libitum*
4	DMH (30 mg/kg in saline), ip.;	Twice weekly for three weeks
	Sulforaphane (10 µmole/kg of SFN) in soy oil, po.	Twice weekly for three weeks
5	DMH (30 mg/kg in saline), ip.;	Twice weekly for three weeks
	Zinc sulphate (227 mg/L) in drinking water;	*ad libitum*
	Sulforaphane (10 µmole/kg of SFN) in soy oil, po.	Twice weekly for three weeks

133x103mm (150 x 150 DPI)



719x393mm (96 x 96 DPI)