Role of Combination Treatment of Aspirin and Zinc in DMH-DSS-induced Colon Inflammation, Oxidative Stress and Tumour Progression in Male BALB/c Mice

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Abstract
Colitis-associated colorectal cancer serves as a prototype of inflammation-associated cancers which is linked with repeated cycles of inflammation and DNA repair deficits. Several preclinical and clinical data reported that aspirin has a chemopreventive effect in colorectal cancer and is associated with dose-dependent side effects. Furthermore, it has been reported that zinc supplementation improves the quality of life in patients undergoing chemotherapy by alteration of colonic cancer cell gene expression. However, explication of the detailed molecular mechanisms involved in the combined administration of aspirin and zinc-mediated protection against colitis-associated colorectal cancer deserves further investigation. For the induction of colitis-associated colorectal cancer, male BALB/c mice were administered 1,2-dimethylhydrazine dihydrochloride (DMH) 20 mg/kg/bw thrice before the initiation of every DSS cycle (3%w/v in drinking water). One week after the initiation of DSS treatment, aspirin (40 mg/kg; p.o.) and zinc in the form of zinc sulphate (3 mg/kg; p.o.) were administered for 8 weeks. Combination of aspirin and zinc as intervention significantly ameliorated DAI score, myeloperoxidase activity, histological score, apoptotic cells and protein expression of various inflammatory markers including nuclear factor kappa light chain enhancer of activated B cells (NFκBp65), cyclooxygenase-2 (COX-2) and interleukin-6 (IL-6); proliferation markers such as proliferating cell nuclear antigen (PCNA), signal transducer and activator of transcription 3 (STAT3) expression significantly decreased, and antioxidant enzymes nuclear factor erythroid 2–related factor 2 (Nrf-2), metallothionein, catalase and superoxide dismutase (SOD) significantly increased as evaluated by immunohistochemistry and western blot analysis.

Keywords Colitis-associated colorectal cancer · Aspirin · Zinc · Inflammation · Apoptosis

Introduction
Colitis-associated colorectal cancer (CACC) is one of the most serious and life-threatening malignancies, which is causally linked to DNA repair defects and inflammatory bowel disease (IBD) [1]. It has already been reported that...
patients with IBD including ulcerative colitis (UC) and Crohn’s disease (CD) are more susceptible to develop colorectal cancer (CRC) compared to the general population [2]. CACC originates from the non-neoplastic inflammatory epithelium and includes inflammation-induced robust genotoxic responses, such as DNA damage and mutations to vital genes (p53, c-src, k-ras, β-catenin and APC), which subsequently drive CACC initiation in IBD cases [3]. Chronic intestinal inflammation contributes to tumour initiation, the first stage of carcinogenesis, by inducing irreparable DNA damage and chromosomal instability, which promote tumour development and progression by enhanced cell proliferation and disturbed apoptosis [4]. Multiple biopsies with various histologic grades found that duration, extent and severity of inflammation are risk factors for neoplasia in ulcerative colitis [5]. The dysfunctional mucus barrier of the intestinal epithelial cells persistently induces the development of colonic inflammation, which can initiate genetic modifications of colonic epithelial cells, leading to neoplastic transformation, aberrant proliferation, angiogenesis and invasiveness [6].

It has been found that aspirin showed a protective effect against dextran sulphate sodium (DSS)-induced colitis in female C57BL/6 mice by increasing the formation of anti-inflammatory and proresolution lipoxins [7]. Furthermore, aspirin reduced sevenfold metastasis incidences in the colorectal cancer of BrafV637E+/Villin-CreERT2/+ mice [8]. Furthermore, aspirin showed a protective effect against colon carcinogenesis in azoxymethane and dextran sulphate sodium-treated CF-1 mice by the modification of histone H3 lysine 27 acetylation [9]. A recent study proves that aspirin reduces the development of colorectal tumours in APCmin/+ mice, and mice were given azoxymethane and dextran sulphate sodium by enrichment of gut microbiota [10]. It has been reported that various pro-inflammatory pathways including nuclear factor kappa B (NF-κB), IL-6/STAT3, cyclooxygenase-2 (COX-2)/PGE2 and IL-23/Th17 promote carcinogenesis by activation of various inflammatory mediators, upregulation of anti-apoptotic genes and stimulation of cell proliferation as well as angiogenesis [11]. According to earlier data, NF-κB is the principal protagonist that controls cell survival and proliferation as well as adhesion molecules that predisposes to intestinal inflammation [12]. Furthermore, it has been reported that Anacardium occidentale L. showed a protective effect against dinitrobenzene sulfonic acid-induced colitis by reducing NF-κB and increasing MnSOD antioxidant expressions [12]. Increased extracellular zinc concentrations showed a protective effect in different stages of colon carcinogenesis as observed in cell lines such as HCT-116, HT-29 and SW620 [13]. Transcriptome analysis revealed an upregulation of specific Zn transporters and zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like protein (ZIP) transcripts in colorectal cancer [14]. Long non-coding RNA Zinc Finger Antisense 1 (ZFAS1) directly targets miR-7-5p in human colorectal cancer and knockdown of the same could inhibit proliferation, migration, invasion and apoptosis [15].

It has been reported that zinc deficiency leads to increased oxidative DNA damage, decreased DNA repair and increased cancer risk through exacerbated ulcerative colitis by the activation of IL-23/Th-19 axis [16]. It has been found that Zn supplementation (35 mg zinc gluconate/day for 30 days) showed a protective effect in ulcerative colitis patients by downregulating the expression of various inflammatory mediators and gene expression of Zn metalloproteins (MT1G and ZIP-14) [17]. Furthermore, patients with IBD tend to have a higher frequency of zinc deficiency such as Crohn’s disease (42.2%) and ulcerative colitis (38.6%) as compared to healthy people [18].

It has been suggested that aspirin prevents epithelial to mesenchymal transition in SW480 colon tumour cells by regulating the Wnt signalling pathway [19]. The chemoprotective role of aspirin, vitamin C and zinc has been reported in the DMH-induced colon carcinoma model, and out of three agents, only zinc supplementation resulted in a better protective effect with improvement in biochemical parameters and 100 percent reduction in tumour incidence [20]. Furthermore, it has been demonstrated that administration of aspirin, zinc and vitamin C at the precarcinoma and carcinoma stages showed an elevated metallothionein level in the colon of DMH-treated rats, as evident from mRNA gene expression and metallothionein protein localisation [21]. It has been reported that combined oral administration of zinc and salicylates in the form of bis(aspirinato)Zn (Zn(aspirnato)2) complex normalised the serum adiponectin level and reduced the high blood pressure in type 2 diabetic condition when administered orally in kk-A(y)mice [22]. Several reports were available regarding the individual protective effects of aspirin and zinc in different experimental models. Considering the synergistic effect of zinc and salicylates and their involvement in several molecular targets, the present investigation has been undertaken to see the possible combination of protective effects of low-dose aspirin with low-dose zinc sulphate intervention in the pathogenesis of DMH + DSS-induced colorectal cancer in male BALB/c mice.

Materials and Methods

Experimental Animals

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC No. 19/44) of the institute, and experiments were performed in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Studies
were performed on male BALB/c mice with a body weight of around 25–30 g obtained from Central Animal Facility, NIPER, Mohali. The animals were housed at approximately 25 °C temperature and humidity of 45–65% with a 12-h light/dark cycle. Free access to a regular pellet diet and purified water were allowed. The animals were acclimatised for 7 days before the initiation of the experiment. Body weight was determined twice weekly, and stool consistency and rectal bleeding were noted every day.

Chemicals

Dextran sulphate sodium salt (MW 30–50 kDa, Cat# 76,203) was purchased from Sisco Research Laboratories. Aspirin (Cat# A5376), zinc sulphate heptahydrate (Cat# 221,376) and 1,2-dimethylhydrazine (Cat# 40,690) were purchased from Sigma-Aldrich Chemicals. Furthermore, FracEL™ DNA Fragmentation Detection Kit (Catalog no. QIA39; Calbiochem, Oncogene Research Product, USA), impress excel staining kit (cat# MP-7601, Vector labs, Burlingame, CA 94,010, USA), mouse lipopolysaccharides ELISA kit (Cat# E2214Mo; Standard Curve Range: 5EU/L – 400EU/L) from elabscience and all other chemicals were purchased from Sigma-Aldrich used to carry out the present experiment.

Study Design

Animals (male BALB/c; 41) were randomly assigned into six groups. Group 1, consists of the control group (n = 5), received normal distilled water. Group 2, as represented ASA+Zn (n = 5), received aspirin (40 mg/kg/day; orally) along with zinc provided as ZnSO4 (3 mg/kg/day; orally) with an interval of 6 h. Group 3 called the positive control group (DMH + DSS; n = 8) and received both DMH and DSS. This group received in a total of three doses of DMH (20 mg/kg; i.p.) during the entire treatment period. The first dose was injected at the initiation of the experiment (0 week), and the 2nd and 3rd doses were administered at the 1st (3 week) and 2nd (6 week) remission periods, respectively. This group also received three cycles of dextran sulphate sodium salt (DSS; 3%w/v) in the entire period of treatment. The 1st DSS cycle was administered after 7 days of the initial DMH administration. The 2nd and 3rd DSS cycles were given for the duration of 7 days, and a gap of 2 weeks was maintained as a remission period in-between the exposure of each DSS administration. Group 4 (n = 7) received DMH + DSS as like group 3 along with aspirin (40 mg/kg/d; orally) from the start of 1st remission cycle and continued till the end of the study. Group 5 (n = 8) received DMH + DSS along with zinc provided as ZnSO4 (3 mg/kg/d; orally) from the initiation of 1st remission cycle and then continued till the termination of the study. Group 6 (n = 9) received DMH + DSS along with aspirin (40 mg/kg/day; orally) combined with zinc in the form of ZnSO4 (3 mg/kg/day; orally) with an interval of 6 h from the initiation of 1st remission cycle and continue till the end of study (Fig. 1).

Rationality of Dose Selection

1,2-Dimethylhydrazine (DMH), a potent colon carcinogen, has a similar tumour incidence to azoxymethane (AOM). Several preclinical evidences reported that administration of DMH (20 mg/kg) or AOM (10 mg/kg) for 1 week and followed by 3 cycles of DSS (3%w/v) develop colitis-associated colorectal cancer (CACC) in the murine model [23] [24] [25]. It has been reported that aspirin at different doses (100 mg/kg, 200 mg/kg and 400 mg/kg; daily) shows protective actions against colon carcinogenesis in humans. Different doses of aspirin in human were equivalent to 20 mg/kg/daily, 40 mg/kg/daily and 80 mg/kg/daily in mice [26, 27]. At the preclinical level, aspirin (20 mg/kg) was not significantly reduce tumour multiplicity. Several articles showed that aspirin at the dose of 325 mg/kg causes gastrointestinal bleeding in humans. The dose of zinc (3 mg/kg) was taken based on its antioxidant and anti-inflammatory activities [28, 29]. Furthermore, studies carried out in different experimental models with aspirin and zinc as intervention agents are shown in Table 1 with citations.

Detection of Aberrant Crypt Foci (ACF) by Methylene Blue Staining

The colon tissue was cut along the longitudinal axis and flushed properly with phosphate buffer saline. The distal colon was fixed flat between filter papers in formalin (10%v/v) for at least 24 h. The fixed colon sections were placed on the microscopic slides with the mucosal surface facing up, stained with methylene blue (0.05%v/v) for 5 min, washed with water to remove all the excess methylene blue stain and observed under a microscope. The aberrant crypts were distinguished from the normal crypts by their increased size, irregular and dilated luminal opening and thicker epithelial lining. The number of aberrant crypts per focus was recorded [29].

Estimation of Disease Activity Index

Disease activity index is the average of percent weight loss, stool consistency and rectal bleeding. The specific scoring criteria were as follows: The score of body weight loss was graded from 0 to 4: 0 was no body weight loss, 1 was 1–5% body weight loss, 2 was 5–10% body weight loss, 3 was 10–15% body weight loss, and 4 was more than 15% body weight loss. Similarly, the score of stool consistency was also among from 0 to 4, that 0 was normal stool, 1 was
moist or sticky stool, 2 was soft stool, 3 was soft stool with mild diarrhoea and 4 was diarrhoea only. The rectal bleeding score was scored using a 0 to 4 scale that 0 was no blood in the stool, 1 was Hemoccult positive, 2 was Hemoccult positive and Visual pellet, 3 was moderate blood, and 4 was gross bleeding [39].

**Estimation of Inflammation by Myeloperoxidase (MPO) Assay**

The colon tissue was homogenised in ice-cold potassium phosphate buffer (50 mM; pH 6.0) containing hexadecyltrimethylammonium bromide (0.5% w/v). The homogenate was frozen and thawed three times, centrifuged at 4000 g for 20 min at 4 °C, and the level of MPO in the supernatant was measured using o-dianisidine. The rate of change in absorbance was measured spectrophotometrically at 460 nm, and MPO activity was expressed as units per 100 mg of protein [29].

**Estimation of Cellular Damage by Histopathological Examination**

For histopathological analysis, colon tissue was fixed in formalin (10% v/v) and then dehydrated in graded concentrations of ethanol and embedded in paraffin. Tissue Sects. (5 μm) were deparaffinized, rehydrated and stained with haematoxylin and eosin (H & E), mounted with DPX mounting media and examined under the microscope (Olympus BX51 microscope, Tokyo, Japan). The sections from each of the animals were evaluated for structural changes. The final histopathological score was calculated as a sum of inflammation, mucosal damage, crypt damage and range of lesions [40]. Alcian blue-Periodic acid Schiff staining was performed to stain colon goblet cells. Briefly, deparaffinized and rehydrated sections were acidified with acetic acid (AA, 3% v/v), followed by incubated with alcian blue solution (1% w/v in 3% v/v AA, pH 2.5). Furthermore, slides were treated with periodic acid (0.5% w/v) and Schiff’s reagent [41]. Processed sections were DPX mounted and examined under the microscope. The percentage of goblet cells areas was quantified using “Image J” software.

**Immunohistochemistry**

Immunohistochemistry was performed using an impress excel staining kit (cat# MP-7601, Vector labs, Burlingame, CA 94,010 USA) according to the manufacturer’s protocol. Briefly, the sections were deparaffinized, hydrated and boiled at 95 °C in citrate buffer for 30 min (10 mM, pH 6) for antigen retrieval. The sections were incubated overnight with primary antibodies of anti-COX-2 (sc-796) and anti-metallothionein (sc-11377) at 1:50 dilution in TBS. Furthermore, slides were treated with secondary antibody and counterstained with haematoxylin. The % immunopositive areas were quantified using “Image J” software.
Table 1  Studies carried out with different dosage regimens of aspirin and zinc in test systems of colorectal cancer

| S. no | Experimental model                      | Aspirin and zinc dosage regimen                  | Test system                                                                 | Observations                                                                 | References |
|-------|----------------------------------------|-------------------------------------------------|-----------------------------------------------------------------------------|------------------------------------------------------------------------------|------------|
| 1     | Colorectal cancer patients             | 81 mg/day; 325 mg/day, p.o                      | Colorectal cancer patients                                                  | Moderate chemo-preventive effect of low-dose aspirin (81 mg/day) decreases colorectal adenomas | [26]       |
| 2     | CRC xenograft model                    | 0, 15, 50 and 100 mg/kg for 2 weeks (which represents 100, 300 and 600 mg equivalent of human dose) | HCT116, SW480, HT29 and LoVo cell lines                                     | Low and medium doses of aspirin (15 and 50 mg/kg) induce apoptosis, suppress tumour growth in CRC xenografts | [30]       |
| 3     | Apc<sup>Min/+</sup> mice instilled with <i>Fusobacterium nucleatum</i> strain Fn7-1 | Animals were provided with AIN-76A diet supplemented with 200 ppm aspirin     | Colon adenomas                                                             | Daily use of aspirin lowers fusobacterial abundance in colon adenoma tissues | [31]       |
| 4     | AOM/DSS mouse model                    | 25 mg/kg/d was administered via drinking water  | Colon                                                                       | Inhibited TXB2 formation, decreased level of PGE2, tumour-associated iNOS-positive macrophages were observed with low-dose aspirin treatment | [32]       |
| 5     | AOM/DSS mouse model                    | 500 µg/kg in feed (which represents 100 mg human dose) | Colon                                                                       | Increased apoptosis by the downregulation of Bcl-2 and Bcl-xl                | [33]       |
| 6     | Apc<sup>Min/+</sup> mice               | 500 µg/kg in feed                              | Colon                                                                       | Decreased β-catenin and PGE2 levels                                          | [34]       |
| 7     | DNBS colitis model                     | Zinc acetate (2, 30 mg/kg., p.o. 3 days)        | Colon                                                                       | Zinc regulates tight junction permeability in ulcerative colitis             | [35]       |
| 8     | DNBS colitis model                     | Zinc sulfate (30, 60 or 120 mg/kg., p.o.)       | Colon                                                                       | Zinc significantly decreases MPO activity and improves body weight in the colitis model | [36]       |
| 9     | DSS colitis model                      | 24 mg/ml Zn as ZnO; p.o                        | Colon                                                                       | Zinc suppresses histology, pathological score and inflammatory indicators such as MPO | [37]       |
| 10    | TNF-α treated Caco-2 cell              | 50 µM ZnSO₄                                    | Caco-2 cell line                                                           | Intracellular Zn mediates intestinal cell survival                           | [38]       |
Terminal Deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) Assay

Colon tissue was fixed in formalin (10% v/v), embedded in paraffin and cut into thin (5 μm) sections with a microtome (Leica RM2145, Germany). TUNEL assay was used for the detection of DNA fragments (Calbiochem, Oncogene Research Product, USA). The assay was conducted according to the manufacturer’s instructions. Image analysis software “Isis” (Carl Zeiss, Axio Imager M1, Germany) was used to count the total cell population and apoptotic cells, and photomicrographs were acquired using charged coupled device (CCD) camera. Apoptotic cells were expressed as a percentage of total cells.

Western Blot Analysis

Radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitor (PI) was used to extract total protein from colon samples. After homogenisation and centrifugation, the protein concentration was measured using the Biuret assay kit. Protein sample, distilled water and loading buffer were mixed in order to adjust the protein concentration to 2 mg/ml. Then, all samples were boiled for 5 min at 98 °C. Protein was resolved on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The resolved protein was transferred onto polyvinylidene difluoride (PVDF)/nitrocellulose membrane. After blocking with (3% w/v) bovine serum albumin (BSA), membrane was incubated with anti-SOD (sc-11407), anti-Nrf2 (sc-722), anti-pNF-κBp65 (sc-33039), anti-Caspase-1 (sc-514), anti-pSTAT-3 (sc-7993-R), anti-STAT-3 (sc-482) and anti-IL-6 (sc-1265) polyclonal rabbit primary antibody, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Enhanced chemiluminescence (ECL) solution and X-ray films were used for the detection of the luminescent signal. Densitometric analyses were quantified by Image J software.

Statistical Analyses

All the values were expressed as mean ± standard error of the mean (SEM) for each group. Statistical difference between the groups was determined by one-way analysis of variance, followed by multiple comparisons with Tukey’s test using Graph pad prism 6 software. The level of statistical significance was set at $p < 0.05$. 

Fig. 2 Combined effect of aspirin and zinc sulphate as an intervention on (A) representative photomacrophraphs showing stool consistency (dotted square), rectal bleeding (dotted circular line) and rectal inflammation (solid square) in male BALB/c mice. All these symptoms were more prominent in DMH + DSS group as compared to controls and intervention groups (DD + ASA, DD + Zn and DD + ASA + Zn). In DMH + DSS group, gross bleeding was observed as compared to the control and ASA + Zn group, whereas symptoms of rectal bleeding were decreased in the individual (DD + ASA; DD + Zn) and combination treatment (DD + ASA + Zn) group as compared to DMH + DSS group. Similarly, stool consistency (loose stools) and rectal inflammation (rectal protrusion) were observed in DMH + DSS group as compared to the control and ASA + Zn group, whereas these symptoms decreased in all the treatment groups (DD + ASA, DD + Zn and DD + ASA + Zn). (B) Graphical representation of disease activity index in male BALB/c mice. All the values are expressed in mean ± SEM (n = 5). ASA, aspirin; Zn, zinc; DMH, 1,2-dimethylhydrazine; DSS, dextran sulphate sodium.
Results

Combined Effect of Aspirin and Zinc on Disease Activity Index

The body weight of DMH + DSS group animals significantly decreased as compared to control and treatment group animals. Rectal bleeding and stool consistency were also higher in DMH + DSS group animals as compared to control and treatment animals. Whereas the combination treatment group (DD + ASA + Zinc) represents increased body weight and lower rectal bleeding and stool consistency as compared to DMH + DSS group and individual treatment (DD + ASA; DD + Zn) group animals (Fig. 2A). Overall DAI score was significantly increased blue and progressing towards the carcinoma stage, whereas all the treatment groups (DD + ASA, DD + Zn and DD + ASA + Zn) exhibited a reduction in the cancer cells with few aberrant crypts. ACF, aberrant crypt foci; ASA, aspirin; Zn, zinc; DMH, 1,2-dimethylhydrazine; DSS, dextran sulphate sodium.

Fig. 3 Representative photomicrographs depicting control and ASA + Zn group representing normal crypts, whereas DMH + DSS (DD), DD + ASA, DD + Zn and DD + ASA + Zn represent aberrant crypt foci (ACF) in murine colonic mucosa stained with methylene blue (0.05%v/v) (indicated with white arrow). DMH + DSS (DD) represents multiple elongated crypts with deeper staining of methylene blue and progressing towards the carcinoma stage, whereas all the treatment groups (DD + ASA, DD + Zn and DD + ASA + Zn) exhibited a reduction in the cancer cells with few aberrant crypts. ACF, aberrant crypt foci; ASA, aspirin; Zn, zinc; DMH, 1,2-dimethylhydrazine; DSS, dextran sulphate sodium.

Fig. 4 Combined effect of aspirin and zinc sulphate as an intervention on (A) representative photomacrographs showing control and ASA + Zn group with no tumour on the distal part of the colon, whereas DMH + DSS (DD) group represents with tumours and combination of aspirin and zinc showed decreased number of tumours on the distal part of the colon. (B) Graphical representation of tumour number in male BALB/c mice. All the values are expressed in mean ± SEM (n = 5). ***p < 0.001 vs. control. **p < 0.01 vs. DMH + DSS group. ASA, aspirin; Zn, zinc; DMH, 1,2-dimethylhydrazine; DSS, dextran sulphate sodium.
### (a)

| Groups                                      | Length of the Colon |
|---------------------------------------------|---------------------|
| Control                                     |                     |
| Drug Perse                                   |                     |
| DMH + DSS                                    |                     |
| DMH + DSS + Aspirin                         |                     |
| DMH + DSS + ZnSO₄                           |                     |
| DMH + DSS + Aspirin + ZnSO₄                 |                     |

### (b)

| Groups                                      | Colon length (mm) |
|---------------------------------------------|-------------------|
| Control                                     | 15                |
| ASA + Zn                                    | 10                |
| DMH + DSS (DD)                              |                   |
| DD + ASA                                    |                   |
| DD + Zn                                     |                   |
| DD + ASA + Zn                               |                   |

### (c)

| Groups                                      | Colon weight/body weight ratio (mg/g) |
|---------------------------------------------|---------------------------------------|
| Control                                     | 1                                    |
| ASA + Zn                                    | 2                                    |
| DMH + DSS (DD)                              |                                       |
| DD + ASA                                    |                                       |
| DD + Zn                                     |                                       |
| DD + ASA + Zn                               |                                       |

### (d)

| Groups                                      | Colon weight/colon length ratio |
|---------------------------------------------|---------------------------------|
| Control                                     | 3                                |
| ASA + Zn                                    | 5                                |
| DMH + DSS (DD)                              |                                 |
| DD + ASA                                    |                                 |
| DD + Zn                                     |                                 |
| DD + ASA + Zn                               |                                 |

### (e)

| Groups                                      | Plasma LPS Level (EULC) |
|---------------------------------------------|-------------------------|
| Control                                     | 0.1                   |
| ASA + Zn                                    | 0.2                   |
| DMH + DSS (DD)                              |                       |
| DD + ASA                                    |                       |
| DD + Zn                                     |                       |
| DD + ASA + Zn                               |                       |

### (f)

| Groups                                      | MDA (nM/mg protein) |
|---------------------------------------------|---------------------|
| Control                                     | 1                   |
| ASA + Zn                                    | 2                   |
| DMH + DSS (DD)                              |                     |
| DD + ASA                                    |                     |
| DD + Zn                                     |                     |
| DD + ASA + Zn                               |                     |

### (g)

| Groups                                      | Myeloperoxidase activity (U/Mg protein) |
|---------------------------------------------|----------------------------------------|
| Control                                     | 3                                    |
| ASA + Zn                                    | 4                                    |
| DMH + DSS (DD)                              |                                      |
| DD + ASA                                    |                                      |
| DD + Zn                                     |                                      |
| DD + ASA + Zn                               |                                      |
in the DMH + DSS group as compared to control and treatment group animals. The combination group represents a significant decrease in DAI score compared to the DMH + DSS group and individual treatment group animals (Fig. 2B).

Combined Effect of Aspirin and Zinc on Intestinal Crypt Foci

Control and ASA + Zn (drug perse) group was observed with normal intestinal crypts. DMH + DSS group was observed with tumours, and aspirin and zinc-treated groups were observed with different stages of colitis-associated colorectal carcinogenesis (Fig. 3 and Supplementary Fig. 1).

Combined Effect of Aspirin and Zinc on Colon Tumour Number

Colon tumour number was significantly increased \( p < 0.001 \) in DMH + DSS group animals as compared to control and treatment group animals, whereas individual treatment group (DD + ASA; DD + Zn) and combination group (DD + ASA + Zn; \( p < 0.001 \)) showed a significant decrease in tumour number as compared to DMH + DSS and individual treatment group animals (Fig. 4).

Combined Effect of Aspirin and Zinc on Morphometric Parameters

Colon length was significantly decreased in DMH + DSS group as compared to control group, whereas combination group (DD + ASA + Zn) significantly increased the colon length as compared to DMH + DSS group and individual treatment group animals (Fig. 5A, B). Organ coefficient (organ weight (g)/animal body weight (g))*100 and colon weight (g)/colon length (cm)*100 were calculated for colon of different groups. DMH + DSS group showed an increase in organ coefficient (\( p < 0.001 \)) and colon weight/colon length ratio \( p < 0.001 \) as compared to control group animals. Combination (DD + ASA + Zn) treated group showed decrease in organ coefficient (\( p < 0.001 \)) and colon weight/colon length ratio \( p < 0.01 \) as compared to DMH + DSS group animals (Fig. 5C, D).

Combined Effect of Aspirin and Zinc on Plasma Lipopolysaccharides (LPS), Malondialdehyde (MDA) Levels and Myeloperoxidase (MPO) Activity

Plasma lipopolysaccharides levels and myeloperoxidase (MPO) activity were significantly increased in DMH + DSS group animals as compared to control \( p < 0.01 \), whereas the combination group (DD + ASA + Zn) significantly decreased plasma lipopolysaccharides levels and myeloperoxidase activity \( p < 0.05 \) as compared to control and individual treatment groups (Fig. 5E, G). Furthermore, there were no significant changes observed in MDA levels in all groups as compared to the control group (Fig. 5F).

Combined Effect of Aspirin and Zinc on Histopathological Analysis

In H&E staining, DMH + DSS group was observed with aberrant crypts, loss of goblet cells (white arrows) and increased cell infiltration (black arrows) as compared to the control group. The combined effect of aspirin and zinc sulphate prevents goblet cell loss and cell infiltration as compared to DMH + DSS group. Histological score of DMH + DSS group was significantly higher as compared to control and treatment groups \( p < 0.001 \), whereas combination treatment (DD + ASA + Zn) showed statistically lower histological score \( p < 0.001 \) as compared to DMH + DSS group (Fig. 6). In alcian blue-periodic acid Schiff staining, DMH + DSS group was observed with loss of goblet cells in mucus region as compared to control \( p < 0.001 \), whereas aspirin and zinc-treated group as well as combination (DD + ASA + Zn) group showed restoration of goblet cells in mucus region as compared to DMH + DSS group \( p < 0.001 \), as shown in Fig. 7.

Combined Effect of Aspirin and Zinc on Immunohistochemistry of Cyclooxygenase-2 (COX-2) and Metallothionein

DMH + DSS group was observed with a significant increase in COX-2 expression and decreased expression of metallothionein as compared to the control group, whereas the combination (DD + ASA + Zn) group significantly decreased the COX-2 expression and restored the metallothionein expression as compared to DMH + DSS group animals (Figs. 8 and 9).

Combined Effect of Aspirin and Zinc on Apoptosis

DMH + DSS group was observed with decreased number of apoptotic cells inside the tumour region, whereas the combination of aspirin and zinc significantly decreased the number of apoptotic cells \( p < 0.001 \) as compared to the DMH + DSS group (Fig. 10).
Fig. 6 Combined effect of aspirin and zinc sulphate as an intervention on (A) haematoxylin and eosin-stained colonic sections, DMH + DSS (DD) group was observed with dysplastic crypts, loss of goblet cell (white arrows) and increased in cell infiltration (black arrows) as compared to control group, whereas the combination of aspirin and zinc sulphate ameliorated the cellular damage induced by 1,2-dimethylhydrazine along with dextran sulphate sodium. (B) Graphical representation of histological score in mice colon. All the values are expressed as mean ± SEM (n = 5). ***p < 0.001 vs. control; ###p < 0.001 vs. DMH + DSS group. ASA, aspirin; Zn, zinc; DMH, 1,2-dimethylhydrazine; DSS, dextran sulphate sodium.

Fig. 7 Combined effect of aspirin and zinc sulphate as intervention on (C) Alcian blue-periodic acid Schiff staining showing goblet cells (red star) on colonic sections; (D) graphical representation of percentage goblet cell area in mice colon. All the values are expressed as mean ± SEM (n = 5). ***p < 0.001 vs. control; ###p < 0.001 vs. DMH + DSS group. ASA, aspirin; Zn, zinc; DMH, 1,2-dimethylhydrazine; DSS, dextran sulphate sodium.
Combined Effect of Aspirin and Zinc on Protein Expression by Western Blot Analysis

DMH + DSS group showed an increase in expression of PCNA, pNFκBp65, Caspase-1, IL-6 and pSTAT3 as compared to the control group, whereas the combination treatment group (DD + ASA + Zn) showed a significant decrease in expression as compared to the DMH + DSS group. Nrf2 and SOD as a marker of antioxidant defence system showed significantly increased expression in DMH + DSS group (tumour region) as compared to the control and treatment groups (Fig. 11).
Discussion

Colitis-associated colorectal cancer (CACC) is a very common malignancy and the second leading cause of cancer-related mortality in the world. Aspirin therapy is well accepted as an analgesic, antipyretic and cardiovascular prophylaxis. Several decades of research provided considerable evidence demonstrating its potential for the prevention of cancer, particularly colorectal cancer [42]. Zinc plays a major role in various physiological processes, acts as an intracellular signalling molecule, repairs DNA damage, inhibits NADPH oxidase, participates in the structure and stability of some enzymes, modulates ATP function and maintains the immune and anti-inflammatory systems [43]. To understand the pathogenesis of colitis-associated colorectal cancer and the preventive effects of combined administration of aspirin and zinc, a novel colitis-related mouse CRC model (a two-stage mouse colon carcinogenesis model) initiated with DMH and promoted by DSS was developed [44]. It has been found that inflammation and ulceration in the colon are responsible for rectal bleeding. It has been reported that mice ingested with 3–10% DSS developed acute colitis and showed the signs of diarrhoea, gross rectal bleeding and weight loss within 6–10 days after the treatment. These symptoms are mainly due to the multiple erosions and the inflammatory changes including crypt abscesses [45]. Aberrant crypt foci (ACF) are considered as earliest prognostic lesions in colorectal cancer progression [46]. These are the clusters of abnormally large colonic crypts present in the mucosal region of the colon [47]. In the present investigation, body weight loss, loose stool and faecal occult bleeding were observed during the initial period, and DAI, which is an indicator of chronic intestinal inflammation, was significantly increased. Later on, rectal swelling, aberrant crypt foci formation, tumour formation and tumour multiplicity were also observed. This inferred that the DSS-induced chronic inflammatory microenvironment favoured the tumorigenesis in the colon leading to CACC progression with time. The milieu of chronic intestinal inflammation plays a vital role in the transformation of colitis into colon carcinogenesis by facilitating cell proliferation, migration and angiogenesis, thereby promoting tumour development, growth and progression. It has been reported that aspirin inhibits the azoxymethane-induced aberrant crypt foci formation in the colons of female CF-1 mice and male F344 rats [48]. Furthermore, zinc supplementation (ZnSO₄; 200 mg/L) in drinking water significantly decreased the aberrant crypt foci number and multiplication, restored damaged histological architecture and mucin production and significantly decreased the expression of pro-inflammatory cytokines in 1,2-dimethylhydrazine-induced colon carcinogenesis model [49]. Administration of aspirin along with
zinc reduced the DAI and tumour progression in DMH-DSS-induced colitis-associated colorectal cancer. Histopathological analysis of colon tissue revealed cell infiltration, crypt damage, epithelium loss and goblet cell loss was observed in DMH + DSS group. On the contrary, all these lesions were less observed in the combination treatment group [50]. From the TUNEL assay analysis, the number of TUNEL positive cells was decreased in the tumour region, whereas surrounding areas around the tumour (normal cells) were observed with more number of apoptotic cells. It has been found that imatinib and its combination with 2,5-dimethyl-celecoxib increase apoptosis in human HT-29 colorectal cancer cells by the upregulation of Caspase-3 enzyme activity [51].

Expression of COX-2 was significantly increased in DMH + DSS group, whereas it was significantly decreased in the aspirin and combination treatment group observed by immunohistochemical analysis. COX-2 is least expressed in normal tissues; however, COX-2 expression is increased in inflammatory and tumour tissues and plays a vital role in inflammation, cell proliferation and differentiation. COX-2 can (i) increase prostaglandins production and modulate the body’s immune response, (ii) inhibit apoptosis in tumour cells and promote cell proliferation, (iii) regulate cell cycle progression, (iv) promote tumour angiogenesis, (v) increase matrix metalloproteinases expression in tumour cells, and (vi) precursors of carcinogenic substances activation [52]. Various preclinical and clinical studies reported that COX-2 inhibitors exerted inhibitory activities during the initiation and post-initiation stages of colorectal carcinogenesis [53]. Furthermore, clinical data of colorectal cancer patients reported that COX-2 mRNA expression was higher in colon tumours compared to the corresponding normal colorectal mucosa [54]. In the present investigation, expression of metallothionein was decreased in DMH + DSS group, whereas
zinc and combination groups were observed with increased metallothionein expression. Metallothioneins (MT) are silenced during tumour progression, mainly through epigenetic changes, and this loss is associated with poor survival of colorectal cancer patients [55]. Zinc ions also potently increase metallothionein expression and are cytotoxic to tumour cells. Various studies reported that MT induction and zinc administration are novel strategies to sensitize colorectal cancer cells to presently utilised chemotherapeutic agents [56]. In the present investigation, PCNA expression was increased in the DMH + DSS group, an indication of cell proliferation as compared to the control group. PCNA is a chief marker reflecting the activity of cell proliferation, which is closely related to the invasion and metastasis of malignant neoplasms and their prognosis [57]. Furthermore, the present investigation represents the increased expression of NFkB and Caspase-1 in the DMH + DSS group as compared to control group animals. As a molecular hub linking inflammation and cancer, NFκB and Caspase-1 have been established as crucial contributors to develop malignant tumours [58]. In earlier reports, it has been found that NF-kB activation supports tumorigenesis by enhancing cell proliferation and angiogenesis, inhibiting apoptosis and promoting cell invasion and metastasis [59]. Furthermore, activation of NF-kB signalling promotes the expression of various pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 that further leads to colonic inflammation and damage [60]. It has been reported that chrysophanol showed protective effect in DSS-induced colitis by suppression of NF-kB/Caspase-1 activation [61].

The present study reported an increased expression of IL-6 and STAT3 expression in the DMH + DSS group, whereas the combination treatment group significantly decreased the expression of IL-6 and STAT3. Furthermore, Nrf2 and SOD expression has been significantly increased in tumour tissue. It has been reported that IL-6/STAT3 is one of the most critical cellular signal transduction pathways known to malfunction in colorectal cancer (CRC) [62]. IL-6 is the key pro-inflammatory cytokine, and its overexpression in colonic mucosa leads to mucosal lesions [60]. It has been found that IL-6 is not only involved in cancer-related inflammation but also contributed to the antioxidant defence system, DNA damage repair, cell proliferation, invasion, metastasis and angiogenesis [63]. The transcription factor STAT3 is an oncogenic protein that is constitutively activated in most tumour cells but not in normal cells. Activation of STAT3 leads to upregulation of the expression of various critical genes involved in cell proliferation and survival, such as the pro-proliferative cyclin D1 and the anti-apoptotic Bcl-2 [64]. It has also been found that aspirin enhanced apoptosis rate by down-regulation of IL-6-STAT3 signalling pathway in azoxymethane (AOM) and dextran sodium sulfate (DSS)-induced colorectal cancer in BALB/c mice [33]. Furthermore, it has been reported that a combination of zinc and melatonin decreased IL-6 levels in 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer in female Wistar rats [65]. Zinc supplementation showed an anti-tumour protective effect in the DMBA-induced breast cancer model by elevating the level of natural killer target cells (NKT) with cytotoxic activity [66]. Under healthy conditions, Nrf2 protects against tumorigenesis and cancer progression by attenuating genotoxic compounds that emerge both intrinsically and extrinsically. However, activation of the Nrf2 defence response can promote the survival of both normal and cancer cells by creating an optimal tumour microenvironment for cell growth [67]. Furthermore, Nrf2 knockout mice were more susceptible to AOM/DSS-induced inflammation-associated colorectal cancer with impaired antioxidant/detoxifying mechanisms coupled with increased expressions of COX-2, 5-LOX and pro-inflammatory metabolites of arachidonic acids [68]. Furthermore, Nrf2 protects tumour cells from oxidative stress, chemotherapeutic agents and radiotherapy, promoting tumour genesis and progression and also metabolic reprogramming to anabolic pathways [69].

From the present study, it can be concluded that combined treatment of low-dose aspirin and low-dose zinc showed a protective effect against DMH + DSS-induced colitis-associated colorectal cancer. Cell proliferation and inflammation have been increased inside the tumour region, as evident from the expression of various proliferation and inflammation markers such as PCNA, IL-6, STAT-3, pNFxBp65 and Caspase-1 p10. Furthermore, an antioxidant defence system has been generated inside the tumour tissue as observed from the expression Nrf2 and SOD. The combination of aspirin and zinc showed a protective effect by targeting inflammation, oxidative stress, cell proliferation and apoptosis. The scientific basis for the combined intervention of aspirin and zinc for the prevention of DMH + DSS-induced colorectal cancer needs to be further strengthened by further preclinical and clinical studies.

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Author Contribution Singothu Siva Nagendra Babu conceived, performed the experiments and analysed the data. Shivani Singla wrote all the sections of the manuscript. G.B. Jena conceived the idea, reviewed the manuscript and administered the project. Finally, all the authors have read and approved the manuscript.

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Data Availability All data and materials support their published claims and comply with field standards. Data will be available on demand.

Declarations

Ethics Approval All institutional and national guidelines for the care and use of laboratory animals were followed. The animal studies were approved by the Animal Ethics Committee of the NIPER SAS Nagar (IAEC/19/44).

Consent to Participate Informed consent is not applicable in the present study.

Consent for Publication All the authors are willing to publish their research work.

Conflict of Interest The authors declare no competing interests.

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