Role of Combination Treatment of Aspirin and Zinc in DMH-DSS Induced Colon Inflammation, Oxidative Stress and Tumor 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 chemo preventive effect in colorectal cancer and is associated with dose dependent side effects. Further, 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 combined administration of aspirin and zinc mediated protection against the 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.) was 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 (NFkBp65), cyclooxygenase-2 (COX-2), 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.

1. 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 been already reported that patients with IBD including Ulcerative colitis (UC) and Crohn's disease (CD) are more susceptible to develop colorectal cancer (CRC) compared to general population [2]. CACC originates from nonneoplastic 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 drives CACC initiation in IBD cases [3]. Chronic intestinal inflammation contributes to tumor initiation, the first stage of carcinogenesis, by inducing irreparable DNA damage and chromosomal instability, which promote tumor development and progression by enhanced cell proliferation and disturbed apoptosis [4]. Multiple biopsies with various histologic grade found that duration, extent and severity of inflammation are risk factors for neoplasia in ulcerative colitis [5]. 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 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]. Further, aspirin reduced 7 fold metastasis incidences in the colorectal cancer of

Braf^{V637E/+}/*Villin-Cre*^{ERT2/+} mice [8]. Furthermore, aspirin showed protective effect against colon carcinogenesis in azoxymethane and dextran sulfate sodium treated CF-1 mice by the modification of histone H3 lysine 27 acetylation [9]. Recent study proves that aspirin reduces the development of colorectal tumors in APC^{min/+} mice and mice given azoxymethane and dextran sulfate sodium by enrichment of gut microbiota [10]. It has been reported that various proinflammatory 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]. Further, it has been reported that *Anacardium occidentale* L. showed protective effect against dinitrobenzene sulfonic acid induced colitis by reducing NFκB and increasing MnSOD antioxidant expressions [12]. Increased extracellular zinc concentrations showed 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 an increased oxidative DNA damage, decreased DNA repair and an increased cancer risk through exacerbated ulcerative colitis by the activation of IL-23/Th-19axis [16]. It has been found that Zn supplementation (35 mg zinc gluconate/day for 30 days) showed 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]. Further, patients with IBD tend to have higher frequency of zinc deficiency such as Crohn's disease (42.2%); Ulcerative colitis (38.6%) as compared to healthy people [18]. Based on the above inferences, it is worthy to explore the combination effect of low-dose aspirin and low-dose zinc sulphate in the pathogenesis of DMH+DSS induced colorectal cancer.

2. Materials And Methods

2.1 Experimental animals

Experimental protocol was approved by 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 body weight 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 12-hour light/dark cycle. Free access to regular pellet diet and purified water was allowed. The animals were acclimatized

for 7 days before the initiation of the experiment. Body weight was determined twice weekly and stool consistency, rectal bleeding was noted every day.

2.2 Chemicals

Dextran Sulphate Sodium Salt (MW 30-50 kDa, Cat# 76203) was purchased from Sisco Research Laboratories; Aspirin (Cat# A5376); Zinc sulphate heptahydrate (Cat# 221376) and 1,2-dimethylhydrazine (Cat# 40690) were purchased from Sigma-Aldrich Chemicals. Further, FragEL™ DNA Fragmentation Detection Kit (Catalog no. QIA39; Calbiochem, Oncogene Research Product, USA); impress excel staining kit (cat# MP-7601, Vector labs, Burlingame, CA 94010, 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.

2.3 Study Design

Animals (male BALB/c; 42) were randomly assigned into six groups. Group 1 consists of Control group (n=5) received normal distilled water; Group 2 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 hours; Group 3 also called Positive control group (DMH + DSS; n=8) received intraperitoneal injection of 1,2-dimethylhydrazine at each recovery period and and three cycles (each cycle; 7 days) of Dextran Sulphate Sodium (3%w/v) in drinking water with the remission period of 14 days; Group 4 (n=7) received DMH + DSS as like group 3 along with Aspirin (40 mg/kg/day; orally) from the start of 1st remission cycle and continued till the end of the study; Group 5 (n=7) received DMH + DSS along with Zinc provided as ZnSO₄ (3 mg/kg/day; orally) from the initiation of 1st remission cycle and then continue 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 hours from the initiation of 1st remission cycle and continue till the end of study (Fig. 1).

2.4 Rationality of dose selection

1,2-dimethylhydrazine (DMH), a potent colon carcinogen, has similar tumor 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. It has been reported that aspirin at different doses (100, 200 and 400 mg/kg; daily) shows protective actions against colon carcinogenesis in humans. Different doses of aspirin in human were equivalent to 20, 40 and 80 mg/kg/daily in mice. At preclinical level, aspirin (20 mg/kg) was not significantly reduced 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. Further, studies carried out in different experimental models with aspirin and zinc as intervention agents shown in Table 1 with citations.

Table 1

Studies carried out with different dosage regimen 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 (81mg/day) decreases colorectal adenomas	[44]
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 aspirin (15 and 50mg/kg) induce apoptosis, suppress tumour growth in CRC xenografts	[45]
3	<i>Apc</i> ^{Min/+} 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	[46]
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	[47]
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	[40]
6	<i>Apc</i> ^{Min/+} mice	500 µg/kg in feed	Colon	Decreased β-catenin and PGE2 levels	[48]
7	DNBS Colitis model	zinc acetate (2, 30 mg/kg., p.o. 3 days)	colon	Zinc regulates tight junction permeability in ulcerative colitis	[49]
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 colitis model	[50]
9	DSS colitis model	24 mg/ml Zn as ZnO; p.o.	colon	Zinc supresses histology, pathological score and inflammatory indicators such as MPO	[51]
10	TNF-α treated Caco-2 cell	50 µM ZnSO ₄	Caco-2 cell line	Intracellular Zn mediates intestinal cell survival	[52]

2.5 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 atleast 24h. The fixed colon sections were placed on the microscopic slides with 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 [19].

2.6 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 that 0 was none body weight loss, 1 was 1% - 5% body weight loss, 2 was 5% - 10% body weight loss, 3 was 10% - 15% body weight loss, 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; Rectal bleeding score was scored using 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 [20].

2.7 Estimation of inflammation by myeloperoxidase (MPO) assay

The colon tissue was homogenized 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 4,000 g for 20 min at 4°C and the level of MPO in the supernatant was measured by 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 [19].

2.8 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 sections (5µm) were deparaffinized, rehydrated and stained with hematoxylin 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 animal were evaluated for structural changes. The final histopathological score was calculated as a sum of inflammation, mucosal damage, crypt damage and range of lesions [21]. 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% in 3%v/v

AA w/v, pH 2.5). Further, slides were treated with periodic acid (0.5%w/v) and Schiff's reagent [22]. Processed sections were DPX mounted and examined under the microscope. The percentage of goblet cells areas were quantified using "Image J" software.

2.9 Immunohistochemistry

Immunohistochemistry was performed by using impress excel staining kit (cat# MP-7601, Vector labs, Burlingame, CA 94010 USA) according to the manufacturer's protocol. Briefly, the sections were deparaffinized, hydrated and boiled at 95°C in citrate buffer for 30 minutes (10 mM, pH 6) for antigen retrieval. The sections were incubated overnight with primary antibodies of anti-COX-2 (sc-796), anti-metallothionein (sc-11377) at 1:50 dilution in TBS. Further, sections were incubated with secondary antibody and counterstained with haematoxylin. The % immunopositive areas were quantified using "Image J" software.

2.10 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Colon tissue was fixed in formalin (10%v/v), embedded in paraffin and cut into thin (5 µm) sections with microtome (Leica RM2145, Germany). TUNEL assay was used for 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 percentage of total cells.

2.11 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 homogenization and centrifugation, the protein concentration was measured using 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.

2.12 Statistical analyses

All the values were expressed as mean $\pm$ 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 $p < 0.05$.

3. Results

3.1 Combined effect of aspirin and zinc on disease activity index

Body weight of DMH + DSS group animals significantly decreased as compared to Control and treatment group animals. Rectal bleeding and stool consistency also higher in DMH + DSS group animals as compared to Control and treatment animals. Whereas 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 in the DMH + DSS group as compared to Control and treatment group animals. The Combination group represents a significantly decrease DAI score compared to the DMH + DSS group and individual treatment group animals (Fig. 2b).

3.2 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 and all the treatment groups were observed with aberrant crypt foci. (Fig. 3 & Supplementary Fig. 1).

3.3 Combined effect of aspirin and zinc on colon tumor number

Colon tumor 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 significantly decrease in tumor number as compared to DMH + DSS and individual treatment group animals (Fig. 4).

3.4 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 & 5b). 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 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 & 5d).

3.5 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 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 & 5g). Further, there were no significant changes observed in MDA levels in all groups as compared to Control group (Fig. 5f).

3.6 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 Control group. Combined effect of aspirin and zinc sulphate prevent the 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).

3.7 Combined effect of aspirin and zinc on immunohistochemistry of cyclooxygenase – 2 (COX-2) and metallothionein

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

3.8 Combined effect of aspirin and zinc on apoptosis

DMH + DSS group was observed with decreased number of apoptotic cells inside the tumor region; whereas combination of aspirin and zinc significantly decreased the number of apoptotic cells ($p<0.001$) as compared to DMH+DSS group (Fig. 10).

3.9 Combined effect of aspirin and zinc on protein expression by western blot analysis

DMH+DSS group showed increase in expression of PCNA, pNF κ Bp65, Caspase-1, IL-6, pSTAT3 as compared to Control group whereas Combination treatment group (DD + ASA + Zn) showed significant decrease in expression as compared to DMH+ DSS group. Nrf2 and SOD as a marker of antioxidant defence system showed significant increased expression in DMH+DSS group (tumor region) as compared to Control and treatment groups (Fig. 11).

4. 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 for cardiovascular prophylaxis. Several decades of research provided considerable evidence demonstrating its potential for the prevention of cancer, particularly colorectal cancer [23]. 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 [24]. 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 [25]. In the present investigation, body weight loss, loose stool, faecal occult bleeding was observed during initial period and DAI, which is an indicator of chronic intestinal inflammation was significantly increased. Later on, rectal swelling, aberrant crypt foci formation, tumor formation, tumor multiplicity were also observed. This inferred that DSS induced chronic inflammatory microenvironment favoured the tumorigenesis in 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 tumor development, growth and progression. Administration of aspirin along with zinc reduced the DAI and tumor progression in DMH-DSS induced colitis associated colorectal cancer. Histopathological analysis of colon tissue revealed cell infiltration, crypt damage, epithelium loss, goblet cell loss was observed in DMH + DSS group. On the contrary all these lesions were less observed in combination treatment group [26]. From the TUNEL assay analysis, number of TUNEL positive cells was decreased in tumor region whereas surrounding areas around tumor (normal cells) was observed with more number of apoptotic cells. It has been found that imatinib and its combination with 2,5-dimethyl-celecoxib increases apoptosis in human HT-29 colorectal cancer cells by the upregulation of Caspase-3 enzyme activity [27].

Expression of COX-2 was significantly increased in DMH + DSS group, whereas it was significantly decreased in 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 tumor

tissues and plays vital role in inflammation, cell proliferation, and differentiation. COX-2 can: (i) increased prostaglandins production and modulate the body's immune response; (ii) inhibit apoptosis in tumor cells and promote cell proliferation; (iii) regulation of cell cycle progression; (iv) promote tumor angiogenesis; (v) increase matrix metalloproteinases expression in tumor cells; and (vi) precursors of carcinogenic substances activation [28]. Various preclinical and clinical studies reported that COX-2 inhibitors exerted the inhibitory activities during the initiation and post-initiation stages of colorectal carcinogenesis [29]. Further, clinical data of colorectal cancer patients reported that COX-2 mRNA expression was higher in colon tumors compared to the corresponding normal colorectal mucosa [30]. In the present investigation, expression of metallothionein was decreased in DMH + DSS group whereas zinc and combination group were observed with increased metallothionein expression. Metallothioneins (MT) are silenced during tumor progression, mainly through epigenetic changes, and this loss is associated with poor survival of colorectal cancer patients [31]. Zinc ions also potentially increase metallothionein expression and are cytotoxic to tumor cells. Various studies reported that MT induction and zinc administration are novel strategies to sensitize colorectal cancer cells to presently utilized chemotherapeutic agents [32]. In the present investigation, PCNA expression was increased in DMH+DSS group indication of cell proliferation as compared to Control group. PCNA is a chief marker reflecting the activity of cell proliferation, which is closely related to invasion and metastasis of malignant neoplasms and their prognosis [33]. Further, present investigation representing the increased expression of NFκBp65 and Caspase-1 in DMH + DSS group as compared to Control group animals. As a molecular hub linking inflammation and cancer, NFκB and Caspase-1 has been established as a crucial contributor in the development of malignant tumors [34]. In earlier reports, it has been found that NF-κB activation supports tumorigenesis by enhancing cell proliferation and angiogenesis, inhibiting apoptosis, and promoting cell invasion and metastasis [35]. Further, activation of NFκB 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 [36]. It has been reported that chrysophanol showed protective effect in DSS induced colitis by suppression of NF-κB/Caspase-1 activation [37].

The present study reported an increased expression of IL-6 and STAT3 expression in DMH + DSS group whereas combination treatment group significantly decreased the expression of IL-6 and STAT3. Further, Nrf2 and SOD expression has been significantly increased in tumor 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) [38]. IL-6 is the key pro-inflammatory cytokine and its overexpression in colonic mucosa leads to mucosal lesions [36]. The transcription factor STAT3 is an oncogenic protein that is constitutively activated in most tumor 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 [39]. 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 [40]. 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 tumor microenvironment for cell growth [41]. Further, 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 proinflammatory metabolites of arachidonic acids [42]. Furthermore, Nrf2 protects tumour cells from oxidative stress, chemotherapeutic agents, and radiotherapy, promoting tumour genesis and progression, and also metabolic reprogramming to anabolic pathways [43].

From the present study, it can be concluded that combined treatment of low dose aspirin and low dose zinc showed protective effect against DMH + DSS induced colitis associated colorectal cancer. Cell proliferation, inflammation has been increased inside the tumor region as evident from the expression of various proliferation and inflammation markers such as PCNA, IL-6, STAT-3, pNFκBp65, Caspase-1 p10. Further, antioxidant defence system has been generated inside the tumor tissue as observed from the expression Nrf2 and SOD. Combination of aspirin and zinc showed 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 further strengthen by further preclinical and clinical studies.

Abbreviations

CACC, colitis associated colorectal cancer; DMH, 1,2-dimethylhydrazine; DSS, dextran sulphate sodium; NFκBp65, nuclear factor kappa light chain enhancer of activated B cells; COX-2, cyclooxygenase - 2; IL-6, Interleukin-6; PCNA, proliferating cell nuclear antigen; STAT3, signal transducer and activator of transcription 3; Nrf-2, nuclear factor erythroid 2–related factor 2; IBD, inflammatory bowel disease; MPO, myeloperoxidase; Caspase-1, cysteine aspartases; SOD, superoxide dismutase; DAI, disease activity index; BSA, bovine serum albumin; MT, metallothionein.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical 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).

Authors Contribution Singothu Siva Nagendra Babu conceived, performed the experiments, analyzed the data and 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.

Informed consent Informed consent is not applicable in the present study.

Plant Reproducibility It is not applicable in the present research.

Clinical Trials Registration It is not applicable in the present research, as all the experiments were carried out in BALB/c mice.

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

Code availability It is not applicable in present research.

Consent to participate It is not applicable in present research.

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

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Figures

Fig. 1.

Figure 1

Schematic representation of experimental design illustrating the treatment protocols and dose regimens for different groups. Control group received normal saline during the entire study time period. ASA + Zn group received aspirin (40 mg/kg, orally) along with zinc sulphate (3 mg/kg, orally) with an interval of 6 hours starting from 1st remission period and then continue, till sacrifice. DMH + DSS (DD) group administered with 1,2- dimethylhydrazine (20 mg/kg, i.p.) thrice during each recovery period along with 3 cycles of Dextran Sulphate Sodium (DSS, 3 %w/v), each cycle consists of 7 days with 7 day recovery period in-between. DD + ASA group received as DMH+DSS group along with aspirin (40 mg/kg, orally) initiated from 1st remission period and then continue throughout the study. DD + Zn group received as DMH+DSS group along with Zinc provided as ZnSO₄ (3 mg/kg/day, orally) starting from 1st remission period and then continue till sacrifice. DD + ASA + Zn received DMH + DSS along with aspirin (40 mg/kg/day, orally) combined with ZnSO₄ (3 mg/kg/day, orally) with an interval of 6 hours starting from 1st remission period and then continue throughout the study. ASA, Aspirin, Zn, Zinc, DMH, 1,2- dimethylhydrazine, DSS, Dextran Sulphate Sodium.

Fig. 2.

Figure 2

Combined effect of aspirin and zinc sulphate as intervention on (a) Representative photomacrographs showing stool consistency, rectal bleeding and rectal inflammation in male BALB/c mice, (b) Graphical representation of Disease Activity Index in male BALB/c mice. All the values are expressed in mean $\pm$ SEM (n =5). ASA, Aspirin, Zn, Zinc, DMH, 1,2- dimethylhydrazine, DSS, Dextran Sulphate Sodium.

Fig. 3.

Figure 3

Representative photomicrographs depicting Control and ASA + Zn group representing normal crypts, whereas DMH + DSS (DD), DD + ASA, DD + Zn and DD + ASA + Zn represents aberrant crypt foci (ACF) in murine colonic mucosa stained with methylene blue (0.05 %v/v) (indicated with white arrow). ACF, Aberrant Crypt Foci, ASA, Aspirin, Zn, Zinc, DMH, 1,2- dimethylhydrazine, DSS, Dextran Sulphate Sodium.

Fig. 4.

Figure 4

Combined effect of aspirin and zinc sulphate as intervention on (a) Representative photomacrographs showing Control and ASA + Zn group with no tumor on distal part of the colon whereas DMH + DSS (DD) group represents with tumors and Combination of aspirin and zinc showed decreased number of tumors on distal part of the colon, (b) Graphical representation of tumor number in male BALB/c mice. All the values are expressed in mean $\pm$ SEM (n =5). ***p<0.001 vs. Control. ###p<0.001 and ##p<0.01 vs. DMH+DSS group. ASA, Aspirin, Zn, Zinc, DMH, 1,2- dimethylhydrazine, DSS, Dextran Sulphate Sodium.

Fig. 5.
(a)

Figure 5

Combined effect of aspirin and zinc sulphate as intervention on (a) Representative photomicrographs showing colon length in male BALB/c mice, (b) Graphical representation of colon length (cm), (c) Percentage colon weight/body weight ratio, (d) Percentage colon weight/colon length ratio, (e) Plasma lipopolysaccharides levels, (f) Malondialdehyde levels, (g) Myeloperoxidase (MPO) activity. All the values are expressed in mean $\pm$ SEM (n=5). ***p<0.001, **p<0.01 vs. Control, ###p<0.001, ##p<0.01 and #p<0.05

vs. DMH + DSS group. LPS, lipopolysaccharides, MDA, malondialdehyde, ASA, Aspirin, Zn, Zinc, DMH, 1,2-dimethylhydrazine, DSS, Dextran Sulphate Sodium, MPO, Myeloperoxidase activity.

Fig. 6.

Figure 6

Combined effect of aspirin and zinc sulphate as intervention on (a) Hematoxylin 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 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 $\pm$ 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.

Figure 7

Combined effect of aspirin and zinc sulphate as intervention on (a) Alcian blue-periodic acid Schiff staining showing goblet cells (red star) on colonic sections, (b) Graphical representation of percentage goblet cell area in mice colon. All the values are expressed as mean $\pm$ 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. 8.

Figure 8

Combined effect of aspirin and zinc sulphate as intervention on (a) Representative photomicrographs showing the immunohistochemical expression of COX-2, (b) Graphical representation of percentage immunopositive area of COX-2. All the values are expressed as mean $\pm$ SEM (n=5). ***p<0.001 vs. Control, ###p<0.001 vs. DMH+DSS group. COX-2, Cyclooxygenase, ASA, Aspirin, Zn, Zinc, DMH, 1,2-dimethylhydrazine, DSS, Dextran Sulphate Sodium.

Fig . 9.

Figure 9

Combined effect of aspirin and zinc sulphate as intervention on (a) Immunohistochemical staining of metallothionein, (b) Graphical representation of percentage immunopositive area of metallothionein. All values are expressed as mean $\pm$ SEM (n=5). ***p<0.001 vs. Control, ###p<0.001 vs. DMH+DSS group. COX-2, Cyclooxygenase, ASA, Aspirin, Zn, Zinc, DMH, 1,2- dimethylhydrazine, DSS, Dextran Sulphate Sodium.

Fig . 10.

Figure 10

(a) Representative photomicrographs showing merged (DAPI + FITC, magnification 200x) of colonic sections, red rectangular area represents TUNEL positive cells, DMH + DSS group was observed with increased number of TUNEL positive cells in surrounding tissues as compared to inside tumor region (b) Graphical representation of percentage TUNEL positive cells. All values are expressed as mean $\pm$ SEM (n=3). ***p<0.001 vs. Control group. TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labelling, ASA, Aspirin, Zn, Zinc, DMH, 1,2- dimethylhydrazine, DSS, Dextran Sulphate Sodium.

Fig. 11.

Figure 11

(a) Representative photomicrographs showing expression of different proteins and their quantification, (b) PCNA expression, (c) IL-6 expression, (d) p-STAT3 expression, (e) T-STAT3 expression, (f) p-STAT3/T-STAT3 ratio, (g) Representative photomicrographs showing expression of inflammatory markers, (h) pNF κ Bp65 expression, (i) Caspase-1 p10 expression, (j) Protein expression of antioxidant markers, (k) Nrf2 expression (l), SOD expression. Combination of aspirin and zinc significantly restored the respective expressions induced by DMH+DSS. All values are expressed as mean $\pm$ SEM (n=3). All values are expressed as mean $\pm$ SEM (n=3). ***p<0.001 vs. Control, ###p<0.001, ##p<0.01 vs. DMH+DSS group. NF κ Bp65, nuclear factor kappa light chain enhancer of activated B cells, COX-2, cyclooxygenase-2, IL-6, Interleukin-6, PCNA, proliferating cell nuclear antigen, STAT3, signal transducer and activator of transcription 3, Nrf-2, nuclear factor erythroid 2-related factor 2, Caspase-1, cysteine aspartases, SOD, superoxide dismutase, ASA, Aspirin, Zn, Zinc, DMH, 1,2- dimethylhydrazine, DSS, Dextran Sulphate Sodium.

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