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ORIGINAL RESEARCH

Chemopreventive Effects of Non-steroidal Anti-inflammatory Drugs in Early Neoplasm of Experimental Colorectal Cancer: an Apoptosome Study

Vivek Vaish · Lalita Tanwar · Jasmeet Kaur · Sankar Nath Sanyal

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Abstract
Purpose Apoptosis is a highly regulated mechanism of cell death where pro-apoptotic proteins and caspases play an important role. Activation of pro-caspases at a definite time is essential to control the whole caspase cascade. Mitochondrion contains some pro-apoptotic proteins, which need to come out in cytoplasm for apoptotic function such as Cytochrome c (Cyt c), while the Bcl-2 protein family works as the guard of mitochondrial membrane and prevents the escape of Cyt c. Once Cyt c is out in cytoplasm, it binds with Apaf-1 (another pro-apoptotic protein also essential for proper cell differentiation) and pro-caspase-9, forming the Apoptosome complex. In this study, the role of two non-steroidal anti-inflammatory drugs (NSAIDs), Diclofenac and Celecoxib, in experimentally induced early neoplasm of colon via apoptosome mechanism had been studied. It has been recognized that the prolonged use of NSAIDs has its effect on reducing the risk of colorectal cancer through apoptotic pathways. However, the role of NSAIDs in respect of apoptosome is not clear.
Methods Western blotting and immunohistochemistry were performed, along with morphological and histological analysis.
Results According to the expression levels of Cytochrome c, Apaf-1, Caspases, and Bcl-2, it was observed that NSAIDs do follow the mitochondrial or intrinsic pathway of apoptosis.
Conclusion The effects of Diclofenac and Celecoxib on the expression of pro- and anti-apoptotic proteins have been observed, which may constitute the mechanism by which the NSAIDs are efficient in controlling the proliferation of neoplasm in the colon.

Keywords cytochrome c · Bcl-2 · Apaf-1 · caspases · apoptosome · NSAIDs

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Introduction

The relative balance between proliferation and apoptosis is responsible for tumor growth. Inhibition of colonic tumor growth cannot be achieved only by decrease in cell proliferation. According to Sinicrope et al. [1], decrease in apoptosis during tumor progression is responsible for colon cancer in humans. Further, in comparison with adenomas, carcinomas have lower rates of apoptosis without any change in proliferation rate. This imbalance in apoptosis and proliferation ratio has a role in tumor development. Initiation of apoptosis in vivo attribute to the antitumor characteristic of non-steroidal anti-inflammatory drugs (NSAIDs) in colon cancer [2, 3]. This has been further confirmed by genetic and pharmacological studies. The anti-tumorigenic properties of NSAIDs are linked with the inhibition of Cyclooxygenase (COX) enzyme, which is a rate-limiting enzyme in Prostaglandin (PG) biosynthesis [4, 5]. COX has two isoforms, COX-1 and COX-2, and both enzymes are role players in the conversion of arachidonic acid (AA) to endoperoxide intermediates, which are further converted to Prostaglandins (PGE_2, PGD_2, $PGF_2\alpha$, and TxA_2) by specific PG synthases [6]. It has been observed that increased arachidonic acid levels and decreased PGE_2 cellular levels are responsible for decreased cellular proliferation and apoptosis. Increased cellular levels of arachidonic acid increase the permeability of mitochondrial membrane and finally release Cytochrome c (Cyt c), a key

Table 1 Occurrence of multiple plaque lesions (MPLs) after 6 weeks of treatment in various groups

Animal groups	No. of MPLs in different region of colon			Total No. of MPLs	No. of rats with MPL/total no. of rats	MPL Incidence (%)	MPL burden	MPL multiplicity
	Proximal	Middle	Distal					
Control	NIL	NIL	NIL	NIL	No MPL	0	NIL	NIL
DMH	8	11	7	27±1.41*	10/10	100	2.7	2.7
DMH + Diclofenac	5	6	4	15± 0.70*/***	8/10	80	1.5	1.8
DMH + Celecoxib	4	5	3	8± 0.63**/****	6/10	60	0.8	1.3

MPL incidence = the percentage of animals having MPLs. MPL burden = the total number of MPLs counted/total number of rats. MPL multiplicity = the total number of MPLs counted/number of MPL bearing rats. The values are mean ± SD of 10 animals.

*$p<0.001$, **$p<0.05$ in comparison to control, ***$p<0.01$, ****$p<0.001$ in comparison to DMH by one way ANOVA.

molecule for mitochondrial apoptosis pathway. Over expression of COX-2 not only generally increases the levels of PGs, which counts for apoptosis inhibition, but increased expression of Bcl-2 has also been reported, which inhibits the release of cytochrome c from mitochondrial membrane and escapes cells from apoptosis [7].

To restore the normal cellular apoptosis mechanism in cancer cells, therapeutic agents (NSAIDs) may initiate mitochondrial pathway of cell death (intrinsic pathway). Under stress conditions, mitochondrion is capable of releasing proapoptotic factor, Cytochrome c (Cyt c), which interacts with apoptotic mediator Apaf-1 (apoptotic protease activating factor) and finally involves procystein aspartate protease 9 (pro-caspase-9) to form the "Apoptosome." Formation of apoptosome converts procaspase-9 to activated caspase-9 or Casp9 [8].

Here, we have studied the role of Apoptosome-mediated cell death in experimentally induced colorectal adenocarcinoma by COX-selective NSAIDS, i.e., Diclofenac (COX-1 and COX-2 preferentially selective) and Celecoxib (COX-2 selective). 1,2-Dimethylhydrazine dihydrochloride (DMH), a potent colon pro-carcinogen, was used to induce colorectal cancer in Sprague-Dawley rats.

Materials and Methods

Chemicals 1,2-Dimethyhydrazine dihydrochloride (DMH) and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac and Celecoxib were a generous gift from Ranbaxy Pharmaceuticals (Gurgaon, India). Primary antibodies against caspase-3, caspase9, Cyt c, Apaf-1, PCNA, Bcl-2, and anti-mouse β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alkaline phosphatase-conjugated secondary antibodies and BCIP-NBT were purchased from Genei Bangalore, India. All other chemicals and reagents used in the present study were of analytical grade and were procured from the reputed Indian manufacturers.

Animal procurement Male Sprague-Dawley rats of body weight between 150 and 200 g were obtained from the inbred population of the Central Animal House, Panjab University, Chandigarh. These were acclimatized to the control diet (rodent chow) and water ad libitum for at least 1 week. Animals were maintained as per the principles and guidelines of the Ethics Committee of Animal Care of Panjab University in accordance with the Indian National Law on animal care and use. The animals were housed three per cage in polypropylene cages

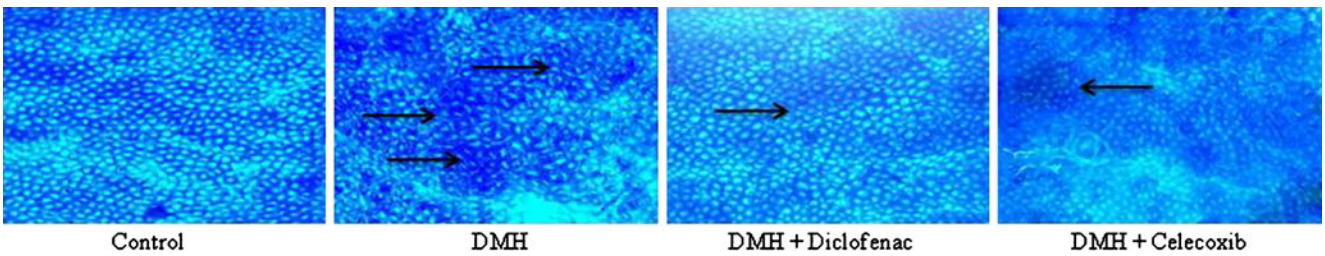

Fig. 1 Surface view of un-sectioned colonic mucosa stained with methylene blue. All the crypts appeared normal in the control group. Aberrant crypt foci were prominently visible in the DMH group (*arrows*), whereas its number decreased with the co-administration of Diclofenac and Celecoxib (×40)

Table 2 Occurrence of aberrant crypt foci (ACFs) after 6 weeks of treatment in various groups

Groups	ACF/colon			
	Small (1−3 crypts)	Medium (4−6 crypts)	Large (>6 crypts)	Total
Control	–	–	–	–
DMH	3	5	8	16±0.51*
DMH + Diclofenac	2	3	1	6±0.69**/***
DMH + Celecoxib	3	1	1	4±0.52**/***

The values are Mean ± S.D. of 10 animals.

*$p<0.001$, **$p<0.05$ in comparison to control, ***$p<0.001$ in comparison to DMH by one way ANOVA.

with a wire mesh top and a hygienic bed of husk (regularly changed) in a well ventilated animal room till the end of the experimental period. The animals were also maintained under a 12-h photoperiod of light and darkness, respectively.

Treatment schedule Animals were sorted into the following groups:

Control Group, Vehicle Treated Animals were administered the vehicle (1 mM EDTA–saline) subcutaneously (sc) in weekly injection and 0.5% carboxymethyl cellulose sodium salt (CMC) per oral (po) daily.

1, 2-Dimethylhydrazine Dihydrochloride (DMH) Group Animals were administered with DMH weekly at a dose of 30 mg/kg body weight (sc). The dose of DMH has been established in our laboratory earlier [9, 10]. DMH was freshly prepared in 1 mM EDTA–saline, pH adjusted to 7.0 using dilute NaOH solution.

DMH + Diclofenac Group Diclofenac was given daily po within its therapeutic anti-inflammatory dose (ED_{50} for rats, 8 mg/kg body weight) to the animals along with the weekly administration of 30 mg/kg body weight of DMH.

DMH + Celecoxib Group Celecoxib was administered po daily (ED_{50} for rats, 6 mg/kg body weight) to the animals along with weekly administration of 30 mg/kg body weight of DMH.

After 6 weeks, animals were kept on overnight fasting with drinking water ad libitum and killed the next day. The animal body weights in all the groups were recorded once a week till the termination of the experiment.

Gross Morphological Observation

The colons were removed and flushed clear with ice-cold physiological saline (NaCl solution, 9 g/L). These were opened longitudinally along the median and laid flat to examine the incidence of macroscopic neoplastic lesions/plaques called the multiple plaque lesions (MPLs). The colons were divided into proximal, medial, and distal segments for the examination.

Aberrant Crypt Foci

The colons were removed, flushed clean, cut open, and divided into different parts as above. After a minimum of 24 h fixation in 10% buffered formalin, the colons were stained with 0.2% methylene blue in Krebs Ringer solution for 5–10 min [11]. The mucosal surface of the colon was evaluated for the number of ACF in the stained colon under ×40 magnification using a light microscope. Enlarged and slightly elevated lesions with increased staining were readily identifiable in comparison to normal adjacent mucosa. These lesions were classified as single enlarged crypts or foci containing two or more abnormal crypts. The sections were then fixed and processed further for histopathological studies.

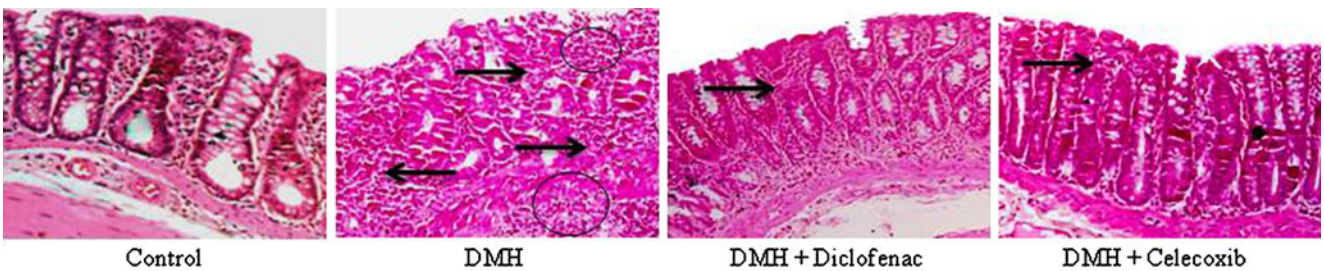

Fig. 2 Histopathological analysis of colonic tissue from various groups in 6 weeks schedule (×100). DMH treated group exhibit severe dysplasia of crypt cells and nuclei enlarged and deeply stained (*arrows* and *circles*). Intact crypt architecture and large number of goblet cells are prominently visible in the sections from the control. DMH + Diclofenac and DMH + Celecoxib groups showed much less dysplastic changes as clearly visible connective tissue layer and less number of mitotic cells with small well stained nuclei were seen. In case of DMH + Celecoxib group, the section is looking more alike the control group as well defined mucosal layer and crypts can be visualized

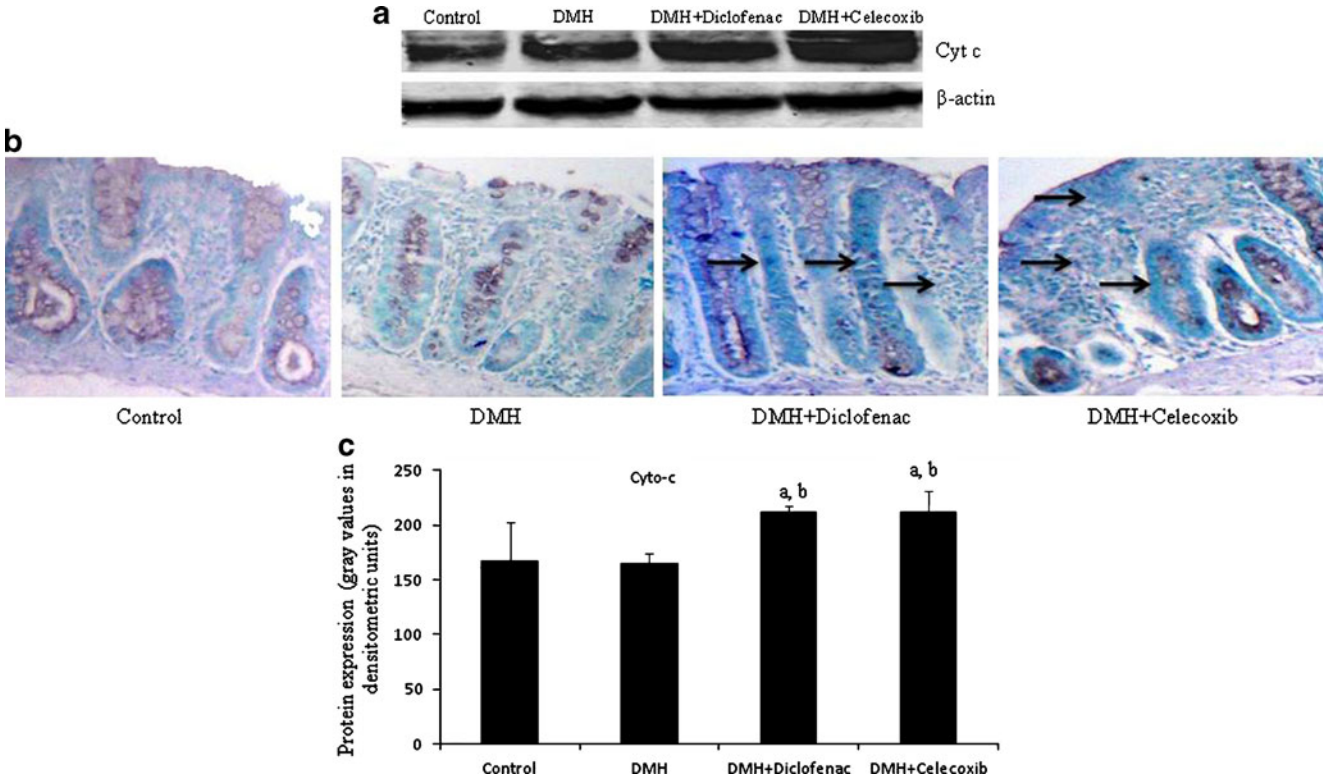

Fig. 3 Western blot analysis of Cyt c showing the effect of DMH and co-administered Diclofenac and Celecoxib on the protein expression (**a**) and its densitometric analysis (**c**). Photomicrographs showing the cytoplasmic expression of Cyt c (*arrows*, dark blue staining) in colon paraffin sections at ×100 under light microscope (**b**). The level of Cyt c expression can be observed higher in case of DMH + Diclofenac and DMH + Celecoxib treated groups with respect to the DMH treated group. The values are mean ± SD of four animals. $^{a}p<0.05$ in comparison to control and $^{b}p<0.05$ in comparison to DMH by one-way ANOVA

Histopathological Analysis

Colon pieces removed from the killed rats were immediately fixed in 10% buffered formalin for 24 h. The tissues were dehydrated in ascending series of alcohol and kept in 1:1 mixture of absolute alcohol and benzene for 1 h. For embedding the tissues in wax, they were kept in benzene for 40–45 min and transferred sequentially to 1:1 benzene and wax mixture at 60°C for 1 h and then pure wax for 6 h at 60°C with two changes. The tissues were embedded in wax and 5-μm-thick sections were cut using a hand-driven microtome and transferred to the egg albumin-coated slides. Sections were then dewaxed in xylene, stained in hematoxylin and eosin, mounted in DPX, and viewed under a light microscope (×100) and photographed.

Immunohistochemical Localization

Five-micrometer-thick paraffin sections were incubated at 60°C for 30 min in an oven for antigen retrieval and deparaffinized in xylene for 30 min. The sections were then gradually hydrated in descending series of alcohol (100%, 90%, 70%, 50%, and 30%). The non-specific staining was blocked by incubating the sections with 2% BSA in phosphate buffered saline (PBS 10 mM, pH 7.2). The sections were then incubated with polyclonal anti-mouse Bcl-2 (1:1,000) and Cyt c (1:1,000) rabbit antibody (Santa Cruz, USA) in a moist chamber for 2 h at 37°C. For negative control, only 1% BSA was added. After incubation with the respective primary antibodies, washing was given with PBS Tween (PBS with 0.05% Tween-20) and PBS successively for 5 min each. The sections were then incubated with the respective secondary antibodies (1:10,000) for 2 h. Sections were washed in the same manner as described above, and the reactive product was developed using BCIP/NBT solution (Genei, Bangalore) under dark conditions. Reaction was terminated by washing with distilled water, counterstained with methyl green, mounted with DPX, and observed under a light microscope.

Western Blot Analysis

Protein samples/nuclear extracts (100 μg) from each treatment group were separated on 10% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose

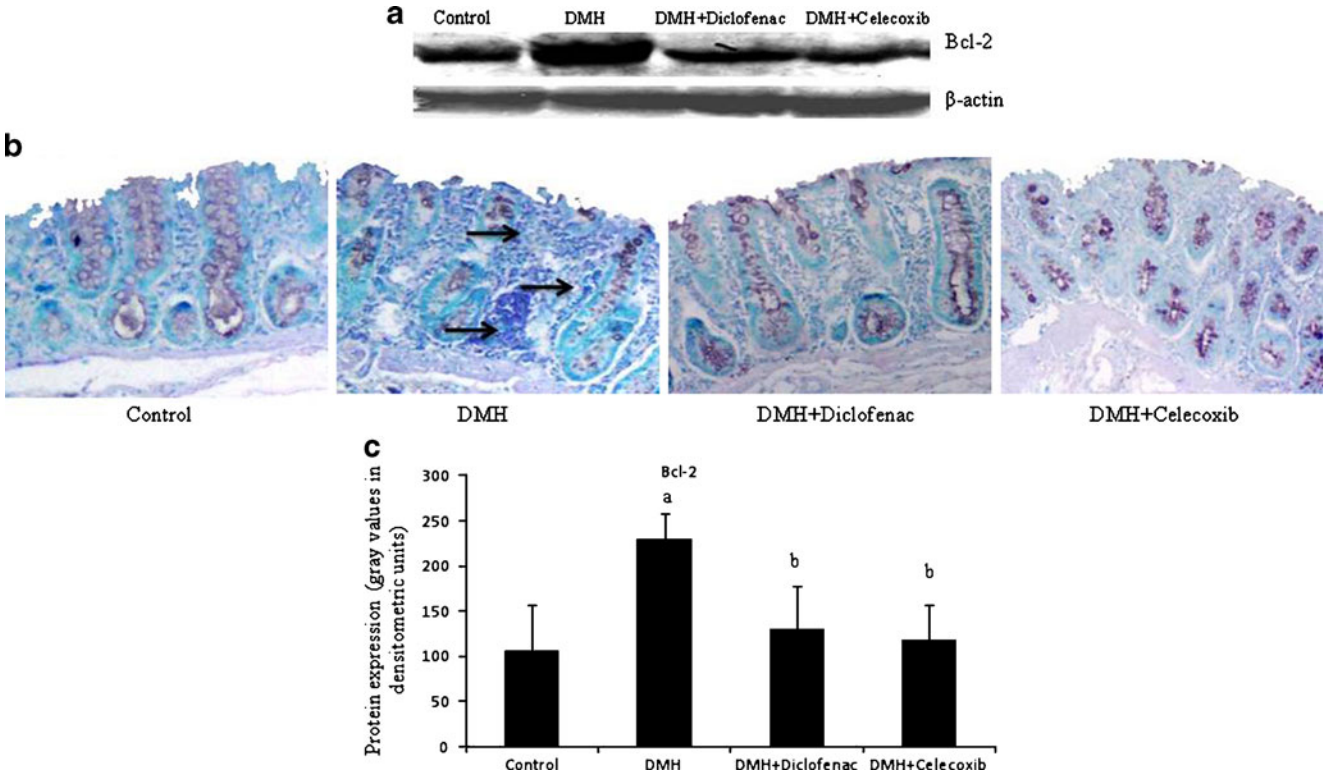

Fig. 4 Western blot analysis of Bcl-2 showing the effect of DMH and co-administered Diclofenac and Celecoxib on the protein expression (**a**) and its densitometric analysis (**c**). Photomicrographs showing the expression of Bcl-2 (*arrows*, dark blue staining) in colon paraffin sections at ×100 under light microscope (**b**). Expression level of Bcl-2 protein can be observed to be higher in DMH treated group with respect to control due to higher rate of proliferation than apoptosis. Lower level of Bcl-2 expression can be observed in case of NSAIDs co-administered groups. The values are mean ± SD of four animals. $^{a}p<0.01$ in comparison to control and $^{b}p<0.01$ in comparison to DMH by one-way ANOVA

membrane (Genei, Bangalore). Immunoblot was prepared using primary antibodies (PCNA:-1:500, Cyt c:-1:1,000, Apaf-1:-1:1,000, Bcl-2:-1:500, β-actin:-1:10,000, Caspase-3: -1:1,000, Caspase-9:-1:1,000) from Santa Cruz Biotechnology and alkaline phosphatase-conjugated respective IgG secondary antibodies (Genei, Bangalore). BCIP-NBT detection system was used to develop the blot. Bands obtained were densitometrically analyzed using Image J software and the density expressed as ratio of densitometric units.

For preparation of cell lysate, colons were removed and rinsed from the different treatment groups after completion of 6 weeks. Total lysates were prepared in fresh ice-cold protein lysis buffer (10 mM Tris, 100 mM NaCl, 5 mM EDTA, 1% Triton-X100, 1 mM PMSF, and 2 mM DTT (pH 8)). The extracts were cleared by centrifugation at 10,000×*g* for 10 min at 4°C.The supernatants were collected as total lysate. For nuclear extract, the nuclei were suspended at 0–4°C in 50 mM NaCl, 10 mM Hepes, pH 7.6, 0.1 mM EDTA, 25% glycerol, and 0.5 mM PMSF and pelleted by centrifugation in an Eppendorf centrifuge at 5,000×*g* for 15 min at 4°C. Resulting nuclear debris was incubated in the same buffer and incubated for 30 min on ice. Centrifuged at 10,000×*g* at 4°C for 10 min, the resultant supernatant was used as the nuclear extract in the present study. Protein concentration was determined by method of Bradford [12].

Statistical Analysis

Data were expressed as mean ± SD of four independent observations for each group. One-way analysis of variance (ANOVA) was done to compare the means between the different treatments using post-hoc comparison by least significant difference (LSD) method. The statistical software package SPSS v14 for windows was used for the purpose. A value of $p<0.05$ was considered significant in the present study.

Results

Morphological Study

Colons were examined by hand held lens for the incidence of multiple plaque lesions (MPLs). There was 100% MPL incidence in case of DMH treated group while 80% and 60% incidence observed in co-administered Diclofenac and

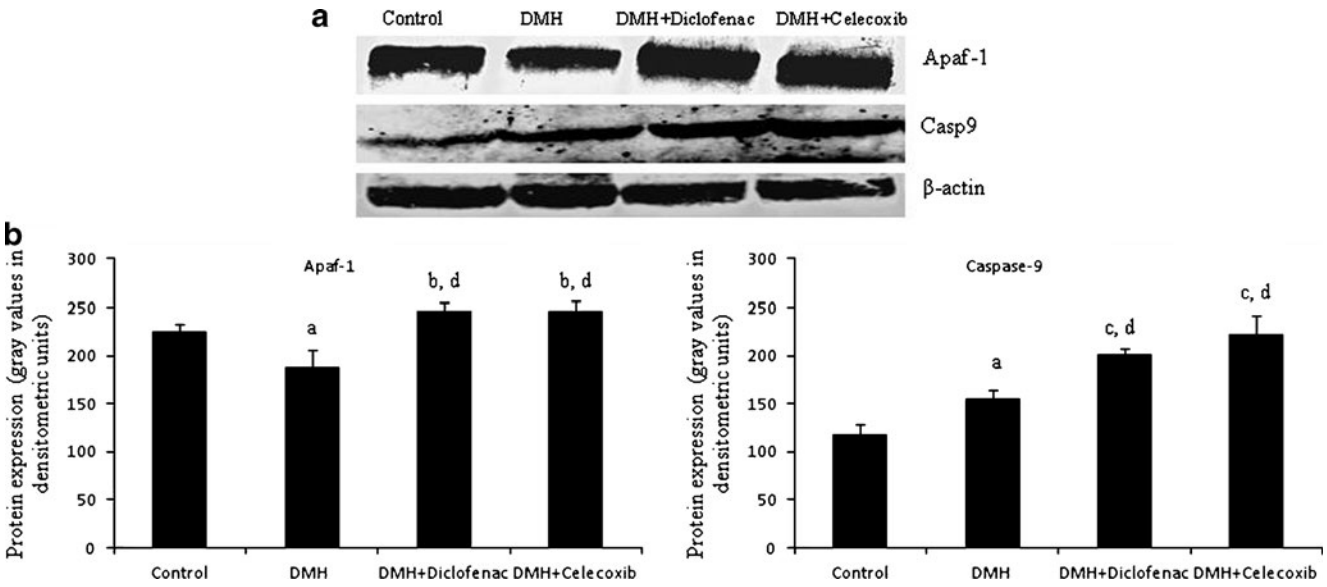

Fig. 5 Western blot analysis of Apaf-1 and Casp9 showing the effect of DMH and co-administered Diclofenac and Celecoxib on the protein expression (**a**) and its densitometric analysis (**b**). Expression of both Apaf-1 and Casp9 can be observed to be higher in NSAIDs co-administered group with respect to the DMH alone group. The values are mean ± SD of four animals. $^{a}p<0.01$, $^{b}p<0.05$, $^{c}p<0.001$ in comparison to control and $^{d}p<0.001$ in comparison to DMH by one way ANOVA

Celecoxib groups, respectively (Table 1). MPLs in rat largely resemble polyps in humans, which occur as outgrowths of tissue either singly if large or in groups if smaller. No MPL was observed in case of the control group.

Aberrant crypts (ACs) and aberrant crypt foci (ACFs) were counted after methylene blue staining under light microscope at ×40. These ACs and ACFs are said to be the preneoplastic lesions with response to carcinogen treatment and are stained darker than the normal crypts (Fig. 1). The microscopic plane of ACs and ACFs was observed to be higher than the normal crypts because of thickening of the epithelial layer. ACs are generally larger than ACFs and are present singly. In case of DMH treated group the number of ACs and ACFs is higher than the other groups with no occurrence in control group (Table 2).

Histopathological Study

After H&E staining, sections were observed for histological changes (Fig. 2). Severe dysplasia with enlarged nuclei was observed in the case of the DMH-treated group with respect to the control. The nuclei were deeply stained and ovoid in shape. Dysplasia was caused by the uncontrolled mitotic divisions. Number of mucin producing goblet cells was also found less with respect to the control group. The connective tissue layer was disturbed due to the dysplastic crypts. Interestingly, there was much less dysplastic change in case of DMH + Diclofenac and DMH + Celecoxib groups, as a clearly visible connective tissue layer and less number of mitotic cells with small well stained nuclei were seen. In case of DMH + Celecoxib group, the section looks more like the control group as well defined mucosal layer and crypts can be visualized.

Mitochondrial Role in Apoptotic Pathway

Initiation of mitochondrial-based apoptosis with the use of NSAIDs supports their chemopreventive and anti-cancerous property. There are many instances that NSAIDs, especially Celecoxib, can activate the mitochondrial pathway of apoptosis. However, in our study, Diclofenac has shown good results in chemoprevention of experimentally raised colon adenocarcinoma.

The level of Cyt c expression was observed higher in case of DMH + Diclofenac and DMH + Celecoxib treated groups with respect to the DMH treated group (Fig. 3a). The same was observed with the immunohistochemical localization of Cyt c protein in paraffin sections (Fig. 3b). Colonocytes are stained deep in case of DMH + Diclofenac and DMH + Celecoxib treated groups with respect to the DMH treated group only.

Expression level of Bcl-2 protein was observed to be higher in DMH treated group with respect to control due to higher rate of proliferation than apoptosis (Fig. 4a). The expression of Bcl-2 was observed to be higher in DMH treated group with respect to the other two NSAIDs co-administered groups. The same was observed with the immunohistochemical localization of Bcl-2 in paraffin sections (Fig. 4b). Comparatively larger, aggregated, and deeply stained colonocytes are visible in DMH treated group rather than smaller, well organized, and lightly stained colonocytes in the control group. Co-

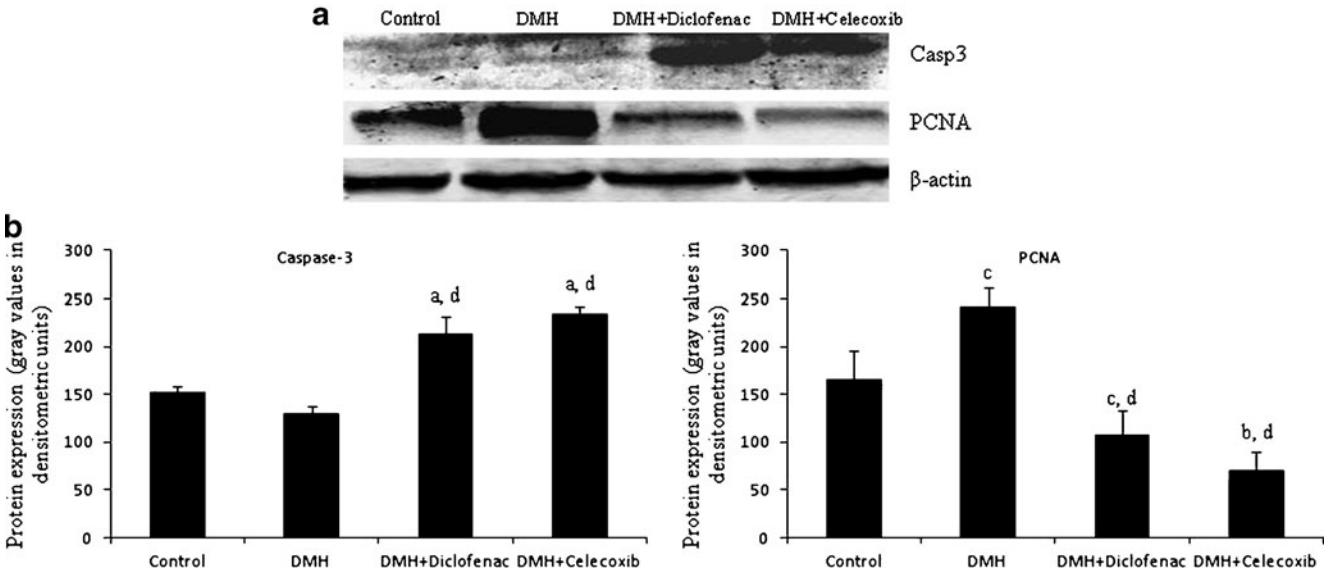

Fig. 6 Western blot analysis of Casp3 and PCNA showing the effect of DMH and co-administered Diclofenac and Celecoxib on the protein expression (**a**) and its densitometric analysis (**b**). Expression of Casp3 can be observed to be higher in NSAIDs co-administered group with respect to the DMH alone group, whereas expression of PCNA is higher in the DMH group with respect to others. The values are mean ± SD of four animals. $^{a}p<0.001$, $^{b}p<0.001$, $^{c}p<0.01$ in comparison to control and $^{d}p<0.001$ in comparison to DMH by one-way ANOVA

administration of NSAIDs with DMH showed lower levels of Bcl-2 expression with respect to the DMH-alone group.

Apoptosome Mechanism of Apoptosis

Western blot analysis of Apaf-1 shows increased expression and, hence, more apoptosis in case of DMH co-administered NSAIDs groups (Fig. 5a). Lower expression level of Apaf-1 in the DMH treated group with respect to the control group indicates the anti-apoptotic and carcinogenic properties of DMH. Expression of Casp9 was observed to be higher in DMH co-administered NSAIDs groups (Fig. 5a).

Nucleus as the Final Target

Once the Apoptosome is formed, the cell is destined to die via apoptosis, where the nucleus is the main cellular organelle and the target for caspase activity. Apoptosome mediated activation of Casp9 finally activated the executioner Casp3. Activation of Casp3 via Apoptosome leads to the activation of a chain of caspases, and this activation is related with nuclear degradation. In Fig. 6a, relatively higher expression of Casp3 was observed in DMH co-administered Diclofenac and Celecoxib groups with respect to Control and DMH groups. This simply confirms the role of Apoptosome and predicts that the cell is ready for apoptotic demise.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein that works as DNA scaffold during DNA repair mechanism. DNA repair mechanism is proportional to the rate of DNA replication because both of the reactions go side by side. This is why the expression of PCNA was observed higher in the case of the DMH treated group with respect to the control due to the higher rate of mitosis essential for tumor progression (Fig. 6a). However, in case of DMH co-administered Diclofenac and Celecoxib groups, the expression is even less than that of the control group, predicting that there is a higher rate of apoptosis, and that is why PCNA was not able to save DNA from degradation and, hence, the nucleus.

Discussion

In this study, we have examined the role of NSAIDs in treatment of early neoplasia of colon via apoptosis based on Apoptosome pathway. We have observed that NSAIDs do follow the mitochondrial or intrinsic pathway for apoptosis which has its relevance in chemo-prevention of colorectal cancer.

Observation of multiple plaque lesions (MPLs) in opened colon suggests that these are the macroscopic sites for the growth of adenocarcinoma. In case of 6-week DMH treatment, they appeared to be like bulges of the epidermis and their increased number, and the large size makes them valuable biomarkers in carcinogenesis. In DMH, co-administered NSAID groups MPLs were small and few in number. Celecoxib has particularly shown better results than Diclofenac with much fewer MPLs.

After division of the colon into three parts, viz., proximal, middle, and distal, ACs and ACFs were counted under ×40, which serves as an important indicator for the evaluation of chemoprevention as it is done with the COX-2 inhibitors in the present study [13]. ACF are characterized as lesions composed of one or multiple enlarged crypts elevated above the surrounding mucosa and are considered as putative preneoplastic lesions of the colon [14].

Bcl-2 protein family are said to be the guards of mitochondrial outer membrane and does not allow the apoptotic factors like Cyt c to escape from there [15–18]. In the present study, it was observed that NSAIDs can alter mitochondrial outer membrane permeability and can commence apoptosis via Cyt c release. The increased expression of Bcl-2 in DMH treated group as compared to the control supports the carcinogenic property of DMH as there is an imbalance between proliferation or differentiation and apoptosis. However, NSAIDs treatment has suppressed the expression of Bcl-2 and, hence, chanced the mitochondrial outer membrane permeability. As can be seen in immunoblot and immunohistochemical analysis, the expression of Bcl-2 differs in all the groups. Change in mitochondrial outer membrane permeability leads towards the release of Cyt c for the commencement of apoptosis. As seen in NSAIDs treated groups, the expression of Cyt c is higher with respect to the DMH treated group. This confirms the role of NSAIDs in Cyt c mediated apoptosis.

The released Cyt c gets bound with Apaf-1 along with procaspase-9 to form the Apoptosome. Apaf-1 is a 130-kDa protein and a key component of apoptosome having an N-terminal Caspase recruitment domain (CARD), a CED-4 homologous domain followed by a large C-terminal having 12 WD-40 repeats, which hinders the recruitment of Casp9 and also Apaf-1 oligomerization. Cyt c can bind to these WD-40 repeats and allow the formation of apoptosome via activating and binding of Casp9 [19–24]. Formation of apoptosome results in caspase–3 (Casp3)-dependent cell death [25]. This reaction gives active Casp9 from procaspase-9 [26]. Higher expression of Casp9 was observed in the case of NSAIDs treated groups with respect to DMH-alone group. However, expression of Casp9 was observed to be higher in DMH treated group than control group, showing its toxicity index on colonocytes.

Expression of Apaf-1 co-relates with the expression of Casp9 and confirms the apoptosome mechanism of apoptosis [27]. The observed higher expression of Apaf-1 in NSAIDs co-administered groups in comparison to the DMH alone group (Fig. 5a) attribute towards the antineoplastic properties of Diclofenac and Celecoxib.

Activation of executer, Casp3, via activated Casp9 leads the cell towards apoptosis through a downstream caspase chain [28–30]. Expression of Casp3 was observed higher in DMH co-administered NSAIDs groups than the DMH-alone treated group. Expression of Casp3 was observed lower in DMH treated group than the control group, predicting that there is a severe imbalance in the ratio of mitosis and apoptosis, which is very much essential for proper cell differentiation and organ development.

PCNA served as a marker for predicting the difference in proliferation [31] and apoptosis among all four groups. PCNA can only be highly expressed in irregularly dividing cell so as to support the DNA while undergoing replication and repair. As seen by the present results expression of PCNA was found to be many folds higher in the DMH treated group.

Conclusion

In this study, we have seen the regulation of proliferation of early neoplasia in colon induced by DMH with the administration of NSAIDs. We have tried to work out the role of mitochondrial and apoptosome pathways in colorectal cancer, and with the supporting results, we can conclude that NSAIDs may exert their effects on mitochondrial or intrinsic pathway of apoptosis, which might proceed via caspase cascade activated by apoptosome.

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