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Original

Chemopreventive response of diclofenac, a non-steroidal anti-inflammatory drug in experimental carcinogenesis

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Abstract

The chemopreventive response was evaluated of non-steroidal anti-inflammatory drug, Diclofenac, a preferential cyclooxygenase-2 (COX-2) inhibitor in 1,2-dimethy-hydrazine (DMH)-induced colon cancer in rat model. The signs of neoplasm were evident in the animals receiving 30mg of DMH per kg body weight in a weekly s.c injection for six weeks. The putative biomarker of carcinogenesis was visible in the form of multiple plaque lesions in DMH treatment and then regression seen in those animals which also received an oral dose of Diclofenac, 8 mg/kg body weight whereas no such macroscopic neoplastic lesions were seen in the animals receiving Diclofenac only or the control animals receiving the vehicle of the drug. Histopathological results showed the presence of early aberrant changes in the form of severe dysplasia and also numerous crypt fissions in the apical surface of the colonic mucosa. A very high expression of COX-2 was seen in the colonic epithelium of DMH-treated rats, as analyzed by immunohistochemistry. Also, the apoptotic events were assessed by terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) assay, where the DMH group shows few number of TUNEL positive cells which dramatically increased in the Diclofenac treatment. The results suggest that Diclofenac could be an effective chemopreventive agent in colon cancer, where perhaps apoptosis plays a very dominant end effect in cancer cell killings.

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Key words: *Colon cancer. Chemoprevention. Diclofenac. Apoptosis.*

RESPUESTA QUIMIOPREVENTIVA DEL DICLOFENACO, UN FÁRMACO ANTIINFLAMATORIO NO ESTEROIDEO EN LA CARCINOGÉNESIS DE COLON EXPERIMENTAL

Resumen

Se evaluó la respuesta quimiopreventiva del fármaco antiinflamatorio no esteroideo, diclofenaco, un inhibidor preferente de la ciclooxigenasa-2 (Cox-2), en el cáncer de colon inducido por 1,2-dimetilhidracina (DMH) en un modelo de rata. Los signos de neoplasia fueron evidentes en los animales que recibieron 30 mg de DMH por kg de peso corporal mediante inyecciones s.c. semanales durante 6 semanas. El biomarcador putativo de la carcinogénesis fue visible en la forma de múltiples lesiones en placas con el tratamiento de DMH y la posterior regresión apreciada en los animales que también recibieron una dosis oral de diclofenaco, 8 mg/kg de peso corporal, mientras que no se vieron tales lesiones neoplásicas macroscópicas en los animales que recibieron solamente el diclofenaco o en los animales control que recibieron el vehículo del fármaco. Los resultados histopatológicos mostraron la presencia de cambios aberrantes precoces en la forma de una displasia intensa y también numerosas fisiones de las criptas en la superficie apical de la mucosa colónica. Se vio una expresión muy elevada de Cox-2 en el epitelio colónico de las ratas tratadas con DMH, al analizarlo por inmunohistoquímica. También se evaluaron los eventos apoptóticos mediante el ensayo *terminal deoxynucleotidyl dutp nick end labeling* (TUNEL), en el que el grupo DMH mostró un pequeño número de células TUNEL positivas, que se incrementó notablemente en el grupo de tratamiento con diclofenaco. Estos resultados sugieren que podría existir un agente quimiopreventivo eficaz para el cáncer de colon en el que, quizás, la apoptosis desempeñe un efecto terminal muy dominante en la muerte de las células cancerosas.

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Palabras clave: *Cáncer de colon. Quimioprevención. Diclofenaco. Apoptosis.*

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed class of pharmacological agents worldwide, having broad clinical utility in treating pain, fever and inflammation.[1,2] Besides the treatment of the inflammatory diseases, NSAIDs in recent times had shown considerable promise as anticancer agents.[3] The anti-inflammatory action of the NSAIDs rest in their ability to inhibit cycloxygenase (COX), which in turn results in a diminished synthesis of the proinflammatory cytokine, the prostaglandin E_2.[4,5] There are two COX isoforms; while COX-1 is constitutively expressed in all tissues, COX-2 is inducible and results due to expression of an early response gene against a variety of stimuli. Expression of COX-2 is increasingly induced during consecutive stages of cancer and the role of the enzyme in colorectal carcinogenesis is well established showing that COX-2 deficiency partly suppressed the Familial Adenomatous Polyposis (FAP) as well as colon cancer.[6] Recently, the use of NSAIDs was linked to the chemoprevention of colorectal cancer,[7] and to a lesser extent breast and lung cancer.[8-10] It is revealed that NSAIDs are effective in reducing the number and size of colorectal polyps in human[11] and animal models,[12] and also can modulate the tumor cell growth in vitro.[13]

Diclofenac [2-(2,6-dicloranilino) phenyl acetic acid] is a highly effective NSAID in reducing inflammation[14] and also there is some evidence to show that Diclofenac is a dual inhibitor of COX-1 and 2.[15] It had shown some anticancer effect in reducing the granulomatous tissue angiogenesis,[16] occurrence of basal cell carcinoma[17] and the inhibition of colon 26 adenocarcinoma development.[18] In view of the reported damage of the gastric mucosa and bleeding due to the antiplatelet effects caused by the inhibition of COX-1[19,20] and also the unexpected cardiovascular side effects due to the COX-2 inhibition alone,[21] it may be an attractive option to use a dual COX-1 and 2 inhibitor like Diclofenac, which could be an as effective agent in the cancer regression as the traditional NSAIDs (COX-1 inhibitor) or specific COX-2 inhibitor (coxibs), but does not overtly manifest the specific pathophysiology of inhibition of either of the two enzyme isoforms. Further, although Diclofenac is known to be a preferentially selective COX-2 inhibitor,[22] there is some evidence that Diclofenac also inhibits the lipoxygenase pathway, thus reducing the formation of the leukotriens, also called the proinflammatory autocoids.[23] There is also speculation that Diclofenac may inhibit phospholipase A_2 as part of its mechanism of action, which may explain the high potency of Diclofenac in the anti-inflammatory disorders.[24]

Therefore, in the present study the effect of Diclofenac in regression of colon cancer was studied in a rat model where chemically induced carcinogenesis and its early changes, and the molecular mechanisms studied. The study included the histoarchitectural changes in the colonic mucosa, expression of COX-2, elucidation of apoptosis and genotoxic damages which are reported with the premalignant changes in colon and chemoprevention with Diclofenac.

Materials and methods

Animals

Male rats (Sprague-Dawley strain) weighing 80-100 g and 2 months old were obtained from the Central Animal House, Panjab University, Chandigarh. These were acclimatized for one week and given standard rodent feed and free access to water. 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.

Experimental Design

Animals were assorted into the following groups and a six week study was conducted:

Group 1: Animals served as controls and were administered vehicle of the drug.

Group 2: Animals were administered with DMH (Sigma, USA) weekly at a dose of 30 mg/kg body weight, subcutaneously (s.c). DMH was freshly prepared in 1mM EDTA-saline (pH 6.5).

Group 3: Diclofenac (8mg/kg body weight) was co-administered p.o daily to the animals along with the weekly dose of DMH. The dose was chosen within the therapeutic anti-inflammatory dose (ED_{50}) for the rats.

Group 4: Diclofenac alone was administered p.o daily (8 mg/kg body weight).

Body weight of the animals was recorded weekly till the termination of the treatment. At the end of the treatment period, the animals were fasted overnight with drinking water *ad libitum* and sacrificed the next day.

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.

Histopathological analysis

Colonic pieces removed from the sacrificed rats were immediately fixed in 10% buffered formalin for

24 hrs. The tissues were dehydrated in ascending series of alcohol and kept in 1:1 mixture of absolute alcohol and benzene for 1 hr. For embedding the tissues in wax, the tissues were kept in benzene for 40-45 min and transferred sequentially to 1:1 benzene and wax mixture at 60ºC for 1 hr and then pure wax for 6 hr at 60ºC with two changes. The tissues were embedded in wax and five micron 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 and photographed.

Immunohistochemical localization

5 µm thick paraffin sections were incubated at 60º 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-goat COX-2 (1:1000 dilution) antibody (Santa Cruz, USA) in a moist chamber for 2 hr at 37º C. For negative control only 1% BSA was added. After incubation with the primary antibody, 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 secondary antibody (1:10,000, Bangalore Genei) for 2 hr. Sections were washed in the same manner as described above and the reactive product was developed using BCIP/NBT solution (Genei, Bangalore, India) under dark conditions. Reaction was terminated by washing with distilled water, counterstained with methyl green, mounted with DPX and observed under a light microscope.

Analysis of Apoptosis (TUNEL assay)

Cells undergoing apoptosis were detected in paraffin tissue sections (5 µm thick) by the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) method, using the *in situ* apoptosis detection kit (Trevigen, USA) according to the manufacturer's recommended protocol. A minimum of 200 cells/field were counted in four randomly selected fields and the index of apoptosis was calculated as the percentage ratio of number of cells with apoptotic nuclei to the total number of cells per field.

Statistical analysis

Data were expressed as mean ± SD of four 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 v10 for windows was used for the purpose. A value of $p < 0.05$ was considered significant in the present study.

Results and discussion

The present study was designed to evaluate the anti-inflammatory efficacy of COX-2 preferentially selective NSAID, called Diclofenac in DMH-induced colon carcinogenesis in rat model. The chemopreventive effects were studied by morphological evaluation of colonic neoplasia, histological analysis of the mucosal epithelium and the immunocytochemical expression of COX-2. The apoptosis in the colonic mucosa was also analyzed by TUNEL assay.

The results in the animal body weight showed a linear growth during the six week treatment schedule (fig. 1),
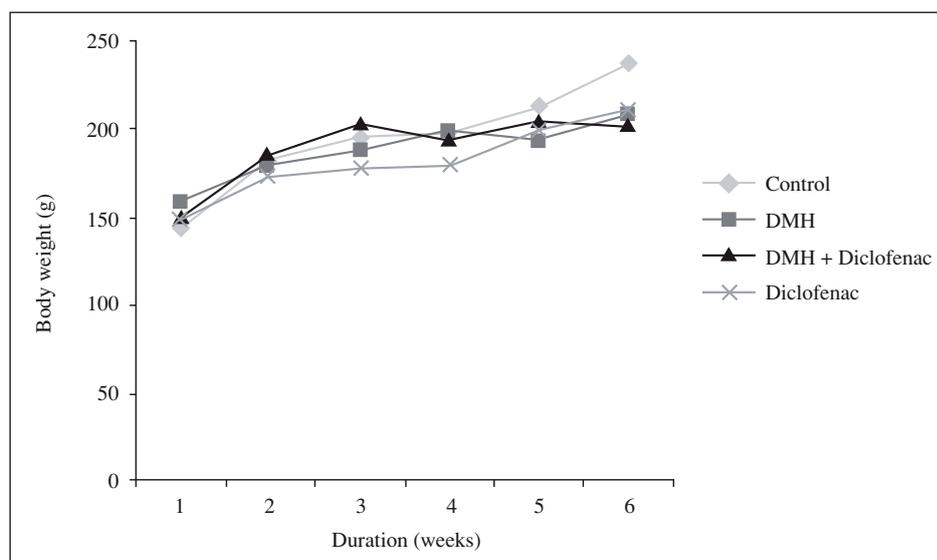

Fig. 1.—Mean body weight profile of control as well as treated groups during a 6 weeks treatment schedule with DMH. No significant change in the body weight was observed with various treatments.
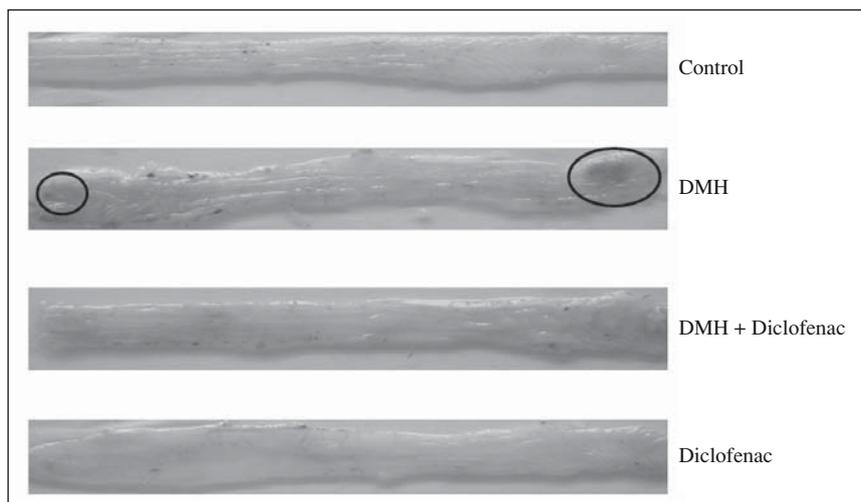
Control

DMH

DMH + Diclofenac

Diclofenac

Fig. 2.—Gross morphology of intact colon showing the occurrence of Multiple Plaque Lesions (MPLs, circled) with DMH treatment (b) and their regression with Diclofenac co-administration (c). Control (a) and Diclofenac only (d) groups showed normal colonic mucosal surface.

with a net weight gain at the end of the study in comparison to their initial weight. No significant change in the body weight between the Control and the different treated groups was observed which shows that the carcinogenic events did not alter the metabolic status of the animals in a significant way and the normal growth pattern achieved.

The colons were grossly examined for the presence of the multiple plaque lesions (MPLs), wherein the neoplastic signs were clearly visible in the DMH-treated animals (fig. 2). MPLs were clearly recognized by the appearance of either raised or non-raised streches of tissues in the form of identifiable tissue growth, often appearing singly or in multiple forms throughout the length of the colon. In DMH + Diclofenac group the number and size of the MPLs were grossly decreased. The Control animals revealed no MPL while very few are observed in the diclofenac only group (table I). Also, the colons of the Diclofenac and DMH + Diclofenac groups appeared almost similar in their gross physical features to that of the Controls. There was also no mortality of the animals noted in any of the groups in the present study. The histopathological analysis in the Control rats of the

colon revealed no signs of malignancy in terms of dysplasia, adenoma or carcinoma and reported the normal epithelium (fig. 3). DMH group revealed the presence of severe dysplasia and numerous malignancy related features along with the development of preneoplastic events such as the occurrence of aberrant crypt foci (ACF) all over the length of the colonic segments, which are the putative biomarkers of colon carcinogenesis.[25] The crypt cells in the dysplasia showed the enlarged nuclei, round or ovoid and deeply stained. Also, the number of mitotic cells is far more and at the same time the number of mucin producing goblet cells markedly reduced. In some of the animals, DMH treatment may not have resulted fully formed adenoma or carcinoma but only severe hyperplasia indicating the onset of the neoplastic and proliferative activities in the mucosal layers. Such sections also revealed the high grade dysplastic crypts at the apical surface through crypt fission, a process which signified the highly proliferative stage of neoplastic mucosal epithelium.[26] In the DMH + Diclofenac treated animals, the regressive effects of the drugs was observed in the DMH-induced carcinogenesis and in the Diclofenac only group the colonic mucosal surface reported a mild hyperplasia

Table I
Chemopreventive response of diclofenac in DMH induced colon carcinogenesis in six weeks treatment groups

| 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	4	2	5	11	8/10	80	1.1	1.375
DMH + diclofenac	2	1	3	6	4/10	40	0.6	1.5
Diclofenac	1	0	0	1	1/10	10	0.1	1

MPLs (Multiple Plaque Lesions).
MPL incidence= the percentage of animals having MPLs.
MPL burden= the total number of MPLs counted/total number of rats.
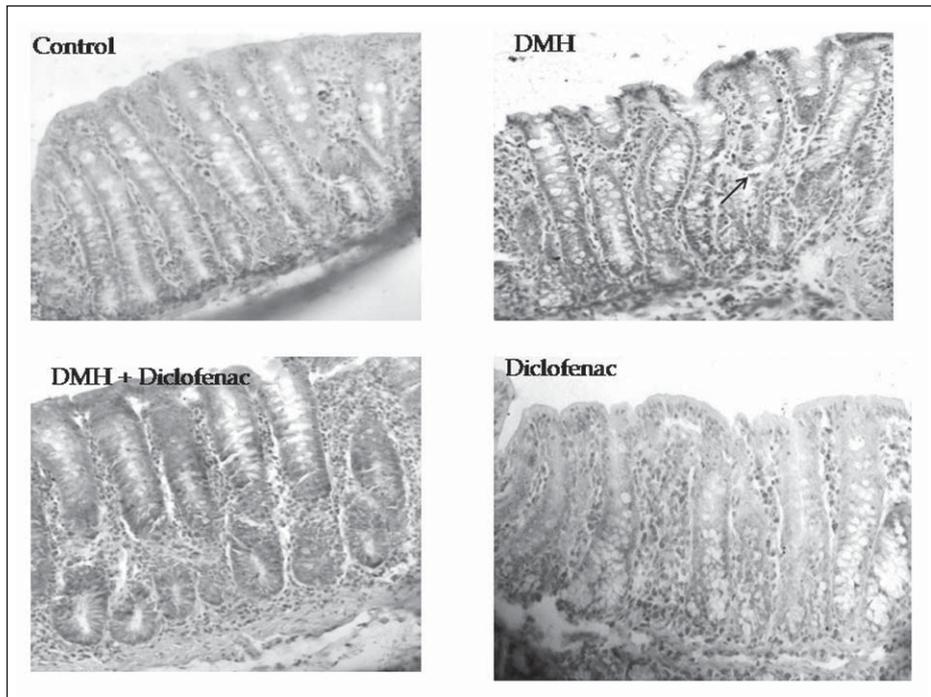MPL multiplicity= the total number of MPLs counted/number of MPL bearing rats.

Fig. 3.—Histopathological analysis of the colonic mucosa after 6 weeks treatment period (200X). a) Control: normal epithelium with no signs of dysplasia, adenoma or carcinoma. b) DMH: severe dysplasia (arrow), loss of mucin producing goblet cells and occurrence of aberrant crypt foci. c) DMH + Diclofenac: moderately hyperplastic d) Diclofenac only: mild hyperplasia, the crypts structures well interspersed with the stromal tissues.

with all the crypt specific structures and other histoarchitectural features nearly intact. Fairly large numbers of goblet cells are visible with the clear mucosa and the stretches of submucosa tissue prominently visible. The crypts are also prominently interspersed in the connective tissue layer, stroma.

The immunohistochemical localization of COX-2 was established with rodent specific antibody in the paraffin sections (fig. 4), which showed a very high cytoplasmic expression of the protein in the DMH group. A much less intense presence of the enzyme in the DMH + Diclofenac group and very mild expression in the Diclofenac only group were also visible but nearly completely absent in the Controls. It was earlier reported that COX-2 is strongly expressed in colorectal cancer than the corresponding non-tumor tissues at
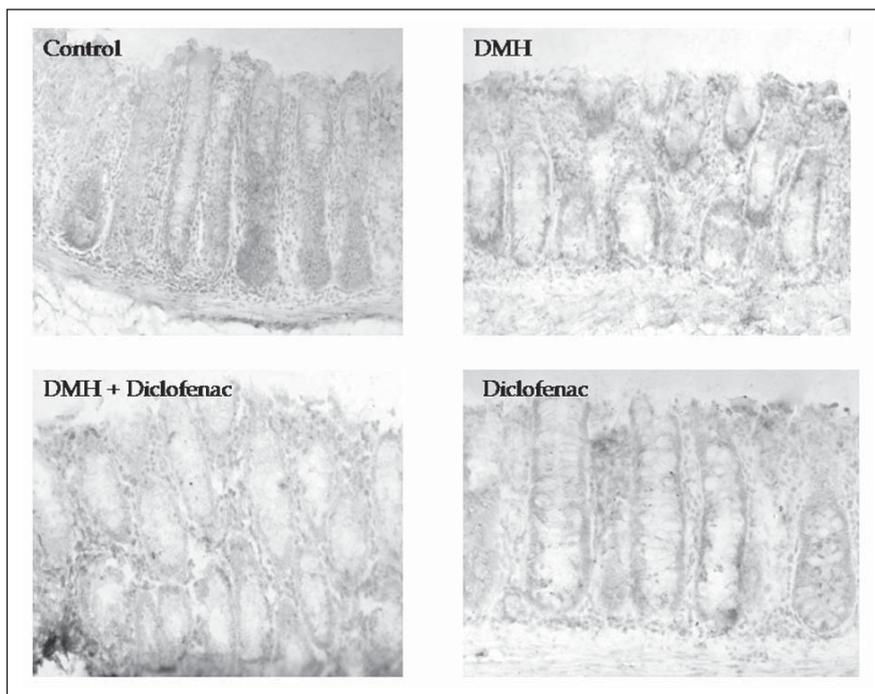

Fig. 4.—Immunohistochemical localization of COX-2 protein in the colonic mucosa (200X). a) Control: Expression of COX-2 absent, b) DMH: High cytoplasmic expression of COX-2, c) DMH + Diclofenac: moderate expression of COX-2 noted d) Diclofenac: mild staining observed, almost similar to the Control sections.
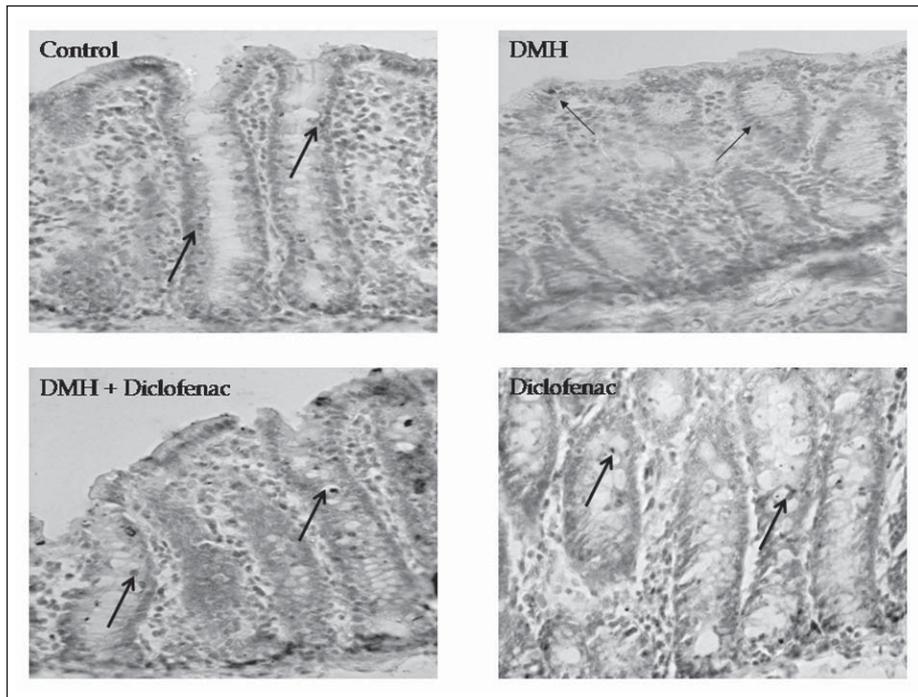
Fig. 5.—TUNEL assay in the colonic mucosa (400X). A marked sign of apoptosis was seen in Control (a) and Diclofenac (d) groups. Sections from DMH treated group (b) showed very few apoptotic/TUNEL positive cells. Co-administration of Diclofenac led to induction of apoptosis in the DMH + Diclofenac group (c), visible by the increase in the number of TUNEL positive cells. TUNEL positive cells are shown by arrows.

both the protein and mRNA level.[27] The COX-2 expression had been progressively increased in cancer showing metastatic potential[28] and that the increased COX-2 levels in human colorectal carcinoma are correlated with both the size and invasiveness of the tumors.[29] Also, a COX-2 over expression had been demonstrated during the recurrence of colorectal carcinoma,[30] especially in the haematogenous metastasis of colorectal cancers.

In the present study, apoptosis was studied using the method of TUNEL, where it often results in cleavage of the nuclear DNA into oligonucleosome size fragments and production of DNA strand breaks which can be revealed by nick end labeling. Only those cells displaying the typical morphological criteria of apoptosis and stained positive by TUNEL assay were counted as apoptotic. A marked sign of apoptosis was seen in the Control rats, while very few TUNEL positive cells seen in the DMH group (fig. 5). Co-administration of diclofenac with DMH resulted in a moderate increase in apoptosis while the Diclofenac only group showed a significant number of TUNEL positive cells (table II). An induction of apoptosis and inhibition of proliferation are widely recognized as potential mechanisms for chemoprevention of colorectal cancer.[31] NSAIDs like Diclofenac are associated with a reduced colon cancer incidence, predominantly by an increased apoptosis.[32]

In conclusion, the results of the present study clearly indicate that a marked sign of apoptosis was seen in the Control and Diclofenac only group as compared to the DMH treated groups, as a normal phenomenon of cellular homeostasis in the colonic crypts. There is accumulating evidence that cellular arachidonic acid bal-

ance may play a key role in regulating apoptosis,[33] for e.g. the diminished production of arachidonic acid as a result of reduced $cPLA_2$-dependent generation or alternately enhanced utilization through COX-2 may deplete the intracellular arachidonate pool, thereby may be attenuating the apoptotic signals and facilitating tumorigenesis.[34] Adenocarcinoma in the human subjects are associated with marked increase in COX-2 expression and evidence from studies with isolated cells in culture or animal models suggest that the prostaglandins produced by COX-2 definitely slow down the rate of apoptosis in cancerous cells.[35] The present results of achieving anticancer effects with Diclofenac surely provides us an option of using not only a dual inhibitor of COX-1 and COX-2 but also at the same time a preferentially selective COX-2 inhibitor, as a very promising chemopreventive agent in colon cancer.

Table II
Percent apoptotic cells in rat colon as observed by TUNEL assay

Groups	No. of apoptotic cells/100 cells
Control	29.75 ± 2.5
DMH	1.75 ± 0.5[c]
DMH + Diclofenac	18 ± 0.6[c/f]
Diclofenac	34 ± 1.4[c]

Values are mean ± S.D. of 4 independent observations by one way ANOVA.
[c]p < 0.001 in comparison to control.
[f]p < 0.001 in comparison to DMH.

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