Research Article

Effect of Ranolazine on 1, 2 - Dimethyl hydrazine induced colon cancer in mice

Ramadevi Pemmireddy[1], Ravikiran Alvala[2], Venkatesh Sama[3], Aparna Sriramoju[1]

[1]*Department of Pharmacology, G. Pullareddy College of Pharmacy, Hyderabad, Telangana, India*

[2]*Department of Biotechnology, G. Pullareddy College of Pharmacy, Hyderabad, Telangana, India*

[3]*Department of Pharmacognosy, G. Pullareddy College of Pharmacy, Hyderabad, Telangana, India*

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Abstract

Objective: The purpose of the present study was to evaluate the effect of Ranolazine on DMH induced colon cancer in mice. **Material and Methods:** Group I (Normal control): Received vehicle p.o. for 7 weeks. Group II (Disease group): Received DMH (20mg/kg) s.c. twice a week for 2 weeks. Group III (Standard control): Received DMH (20mg/kg) s.c. twice a week for 2 weeks and 5-fluoro uracil (20 mg/kg) i.p. for 14 consecutive days. Group IV (Ranolazine 10): Received DMH (20mg/kg) s.c. twice a week for 2 weeks and Ranolazine (10 mg/kg) p.o. for twice a week for 7 weeks. Group V (Ranolazine 20): Received DMH (20mg/kg) s.c. twice a week for 2 weeks and Ranolazine (20 mg/kg) p.o. twice a week for 7 weeks. At the end of the treatment period, all the animals were sacrificed by cervical dislocation and the colons were isolated. Finally, the colonic tissues were used for quantification of aberrant crypt foci, biochemical estimations (MDA, GSH, Catalase and SOD) and histological examination. **Results:** Mice treated with DMH showed high incidents of aberrant crypt foci along with malondialdehyde (MDA) and decreased GSH, SOD and Catalase levels. Histopathalogical evaluation of colon in DMH treated mice also showed dysplasia, inflammation and focal congestion in sub-mucosa and muscularis layers. The treatment with Ranolazine reversed all the above effects and restored to the normal level. **Conclusion:** In conclusion, the present study suggests that treatment with Ranolazine significantly decreased colonic ACF probably due to dysregulation of cancer cell progression.

***Keywords*:** 1, 2-dimethylhydrazine, Ranolazine, Aberrant crypt foci, Colon cancer

Introduction

Colon cancer is the third most common cancer and fourth most frequent cause of cancer deaths worldwide. Colon cancer begins as a benign polyp, a growth of tissue that starts in the lining and grows into the center of the colon. The benign polyp can develop into an advanced adenoma with high-grade dysplasia eventually progressing to an invasive cancer.

Age, genetics, dietary components, smoking and alcohol consumption personal history of colon polyps, family history of cancer and lack of exercise are the major risk factors associated with colon cancer (Tsong et al., 2007). Ethnic and geographical studies suggest that environmental factors may play an important role in colorectal cancer with rates higher in North American and northern Europe, lower in southern Europe, and much lower in Asia and Africa (Doris et al., 2011).

The main symptoms of colon cancer include change in bowel habits, diarrhea, constipation, or feeling that the bowel does not empty completely, bright red or very dark blood in the stool, discomfort in the abdomen including frequent gas pains, bloating, fullness and cramps, un explained weight loss, constant tiredness or fatigue, unexplained iron-deficiency anemia.

The most common treatment for colon cancer includes surgery (colectomy, polypectomy), radiation therapy and chemotherapy. Various chemotherapeutic drugs are used to treat colon cancer like 5-flourouracil, leucovorin, irinotecan, bevacizumab and cetuximumab depending on the stage of the disease but there is no specific therapy till date (National Cancer Institute; Physician Data Query (PDQ); Colon Cancer Treatment, 2009). As a result of the limitations of conventional therapy like life-threatening diarrhea, neuropathy, Immunosuppression, etc., there is a need to

Address for Corresponding Author:
Ramadevi Pemmireddy
Assistant Professor, Department of Pharmacology, Sree Dattha Institute of Pharmacy, Hyderabad, Telangana, India
E-mail: ramadevipemmireddy002@gmail.com

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develop novel and safe therapeutic agents to treat colon cancer.

Colon carcinogenesis is known to be a pathological consequence of persistent oxidative stress, resulting in DNA damage and mutations in cancer associated genes in which the cellular over production of reactive oxygen species (ROS) have been implicated (Boyle and Langman, 2000; Roberto et al., 2004).

DMH is a toxic environmental pollutant which was reported as a specific colon procarcinogen. Animal studies showed that experimental colonic tumors induced by DMH were of epithelial origin with a similar histology, morphology and anatomy to human colonic neoplasms (Xu Dong Jia and Chi Han, 2000; Khan and Sultana, 2011). This procarcinogen could thus provide an adequate model for studying colon cancer. DMH is believed to form active intermediates including azoxymethane and methylazoxymethanol is decomposed to form methyldiazonium ions, which methylate cellular components. DMH also produces free radicals that induce oxidative DNA damage in the liver and colon. Damage to DNA from ROS is a consequence of oxidative stress, and several oxidative DNA adducts (Mitchell, 2005; Aranganathan and Nalini, 2009).

ACF in the colonic mucosa have been hypothesized to represent precursor lesions of chemically induced colon cancer. ACF system can be used as a short-term bioassay to screen potentially new chemopreventive agents and to evaluate the effect of protective factors at a very early stage of carcinogenic process. They are defined as crypts that have (i) altered luminal openings, (ii) exhibit thickened epithelia, (iii) are larger than adjacent normal crypts and (iv) are microscopically elevated (Hang et al., 2007).

Ranolazine is the first new antianginal drug developed over 20 years (Lars and Samuel, 2013). Ranolazine is the voltage-gated sodium channel blocker (Samuel and Lars, 2012). VGSC activity enhances cancer cell invasiveness (Yun et al., 2011). Indeed blockers of 1NaP are very effective in suppressing cancer cell invasiveness in vitro. Already this drug has been used in the treatment of cardiac angina and there are no issues of dosage, unacceptable side effects/long term use (Djamgoz and Onkal, 2013). However, till date, no studies have been reported the antitumorogenic effect of Ranolazine against DMH induced colon cancer in mice.

Materials and Methods

Experimental animals

Male Swiss albino mice weighing 20-22g were purchased from National Institute of Nutrition (NIN), Hyderabad. They were placed individually in clean, transparent polypropylene cages with free access to food and water with 12: 12 hr dark/light cycle is followed. They were acclimatized for a period of one week.

All the experimental procedures were carried in accordance with the committee for the purpose of control and supervision of experiments on animals. The study was approved by the Institutional Animal Ethical committee, G. Pulla Reddy College of pharmacy, Hyderabad (GPRCP/IAEC/11/13/3/PCL/AE2-mice-M/F-30).

Chemicals

1,2-dimethyl hydrazine (procured from sigma Aldrich), Ranolazine is obtained as a generous gift sample from MSN laboratories, Hyderabad, 5-Fluro Uracil is purchased from SD fine chemicals, TCA was purchased from HIMEDIA. Ellman's reagent, TBA and Dopamine HCl were purchased from Sigma Aldrich, USA,Salts required for the preparation of buffers were purchased from SD fine chemicals.

Experimental design (Wang et al., 2004; Ilan et al., 2010; Karthikkumar et al., 2013; Nahedet al., 2013).

Thirty Swiss albino mice were divided into 5 groups; each group consists of 6 animals. Colon cancer was induced by injecting 20 mg/kg s.c in saline twice a week for 2 weeks (Parthasarathy, 2011; Vinoth et al., 2014).

Group 1 (Normal control): Received saline s.c. twice a week for 2 weeks.

Group 2 (Disease control): Received DMH (20mg/kg, s.c.) twice a week for 2 weeks.

Group 3 (Standard): Received DMH (20mg/kg, s.c.) twice a week for 2 weeks and 5-fluorouracil (20mg/kg.i.p.) once daily for 2 weeks.

Group 4 (Disease treated): Received DMH (20mg/kg, s.c.) twice a week for 2 weeks and Ranolazine (10 mg/kg, p.o) twice daily for 7 weeks.

Group 5 (Disease treated): Received DMH (20mg/kg, s.c.) twice a week for 2 weeks and Ranolazine (20 mg/kg, p.o) twice daily for 7 weeks.

At the end of treatment period (8 weeks), all the animals were sacrificed and the colons isolated. Finally, the colonic tissue was used for quantification of aberrant crypt foci, biochemical estimations (MDA, GSH, Catalase, SOD) and histological examination. Detailed experimental design was depicted.

Evaluation parameters

Determination of aberrant crypt foci

Aberrant crypt foci (ACF) were counted by the method of Bird, isolated colons were flushed with phosphate buffered saline and opened along the longitudinal median axis, and

fixed flat between two pieces of filter paper in 10% neutral buffered formalin for 24 h. Colon tissues that were fixed in formalin were removed and stained with 0.2% Methylene blue (0.2 g in100 ml distilled water) for 5 minutes then briefly rinsed with distilled water, they were then placed on a microscope slide and observed under a light microscope at 40X magnification. ACF were distinguished by their slit-like opening, darkly stained, size and pericryptal zone and slight elevation compared to normal crypts (Hang et al.., 2007).

Determination of malondialdehyde (MDA) concentration

100 mg of tissue was weighed appropriately and homogenized in 5 ml of buffer solution with remi motor at a speed of 2500 rpm for 2 minutes in ice cold surrounding environment. The homogenate is centrifuged at 12000 rcf at 4°c for 20 minutes.

The level of malondialdehyde was determined by method of Ohkawa et al. (1979). A volume of 500µl of supernatant of 2% tissue homogenate in 0.15mol/L KCl was mixed with 200µl of 8.1% SDS and then incubated at room temperature for 5 min. then volume of 1.5ml of 20% acetic acid (pH 3.5) & 1.5ml 0.8% thiobarbituric acid was added and then the reaction mixture was heated at 95ºC for 90 minutes and then 1ml of distilled water & 5ml butanol /pyridine (15:1) solution was added under agitation using a vortex after cooling the mixture. This solution was centrifuged at 1000xg for 15min & the resultant colored layer was separated and measured at 532nm using spectrophotometer.

$$\text{Concentration of MDA} = \frac{\textbf{Absorbance}}{\textbf{L}} \textbf{x} \frac{\textbf{1}}{\boldsymbol{\varepsilon}} \textbf{x D}$$

Where,

L: Light path (cm)

ε: Extinction coefficient ($1.56 \times 10^5 M^{-1}.Cm^{-1}$)

D: Dilution factor

Determination of superoxide dismutase (SOD)

SOD level was determined by the procedure described by Misra et al., (1972). A volume of 0.5ml of supernatant (2% tissue homogenate in 0.1 M phosphate buffer, pH 7.4) was taken and 1.5ml of carbonate buffer (pH 10.2) and 0.5ml of 0.1mM EDTA was added. A volume of 0.4ml of epinephrine was added just before taking the optical density. Optical density was measured spectrophotometrically at 480 nm.

$$A = \varepsilon bc$$

Where,

A= Absorbance

ε= Molar absorptivity coeffeicient ($4020 M^{-1}Cm^{-1}$)

b= Path length (cm)

c= Concentration

Determination of catalase

Catalase (CAT) activity was determined by the method of Elzbietaet al., (2005). 0.1ml of supernatant of 2% tissue homogenate in 0.1M phosphate buffer pH 7.4 was added to cuvette containing 1.9ml of 50mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0ml of freshly prepared 30mM H_2O_2 The absorbance was read at 15 and 30 seconds at 240 nm.

$$\text{Activity of Catalase (K/ml)} = \frac{Vt}{vs} x \frac{2.303}{\Delta t} x \log \frac{A1}{A2} x 6$$

Where,

Δt: (time 2 - time 1) = 15 seconds.

A_1: First absorbance at 15 seconds.

A_2: Second absorbance at 30 seconds.

V_t: Total volume = 3 ml.

Vs: Sample volume = 2 ml.

K: First order rate constant. It expresses the activity of Catalase.

Determination of reduced glutathione (GSH) levels (Ellman)

Glutathione GSH was analyzed according to Ellman's method (Davidson and Hird, 1964). Tissue weighing 10 mg was taken and homogenized in 0.1M phosphate buffer pH 7.4. A volume of O.5 ml of tissue homogenate was added with equal volume of 20% Tricholoro acetic acid containing 1mM EDTA to precipitate the tissue proteins. Mixture was allowed to stand for 5 minutes and then centrifuged at 200 rpm for 10 minutes. Supernatant (200µl) was taken in fresh tubes and then the test tubes are filled with 1.8ml of the Ellman's reagent (0.1mM) which was prepared in 0.3M phosphate buffer with 1% sodium citrate solution. After completion of total reaction, solution was read at 412 nm against blank.

$$\text{Concentration of GSH} = \frac{\textbf{Absorbance}}{\textbf{L}} \textbf{x} \frac{\textbf{1}}{\boldsymbol{\varepsilon}} \textbf{x D}$$

L: Light bath (cm)

ε: Extinction coefficient ($14150 M^{-1}.Cm^{-1}$)

D: Dilution factor.

Histopathological evaluation

The colon was excised. Flushed with saline, cut open longitudinally along the main axis, and then again washed with saline, these colonic sections were fixed in 10% buffered formalin for at least 24 h and after fixation, the specimens were dehydrated in ascending grades of ethanol, Cleared in

benzene, and embedded in paraffin wax. Blocks were made and 5µm thick sections were cut from the distal colon. The paraffin embedded colonic tissue sections were deparaffinized using xylene and ethanol. The slides were washed with phosphate buffered saline (PBS) and permeabilized with permeabilization solution (0.1 M citrate, 0.1% Triton X-100). These sections stained with hematoxylin and eosin and were observed under light microscope at 40X magnification to investigate the histoarchitecture of colonic mucosa (Sengottuvelan et al., 2009).

Statistical analysis

Results are expressed as mean±SEM. statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test as post hoc by using Graph Pad prism software (5.03 version).

Results

Aberrant crypt foci

Aberrant crypt foci formation is an indicator of colon carcinogenesis. ACF are clusters of tube like glands distinguished by their slit like opening, darkly stained, and slightly elevated compared to normal crypts. Table 1 summarizes the effect of Ranolazine on ACF formation. In the disease control group the number of aberrant crypts will be more due to induction of colon carcinogenesis by DMH. In the group treated with Ranolazine at 10 mg/kg, the number of aberrant crypts has been reduced to some extent, where as in the group treated with Ranolazine 20 mg/kg, the number of aberrant crypts are significantly reduced when compared with disease and standard group (Figure 1).

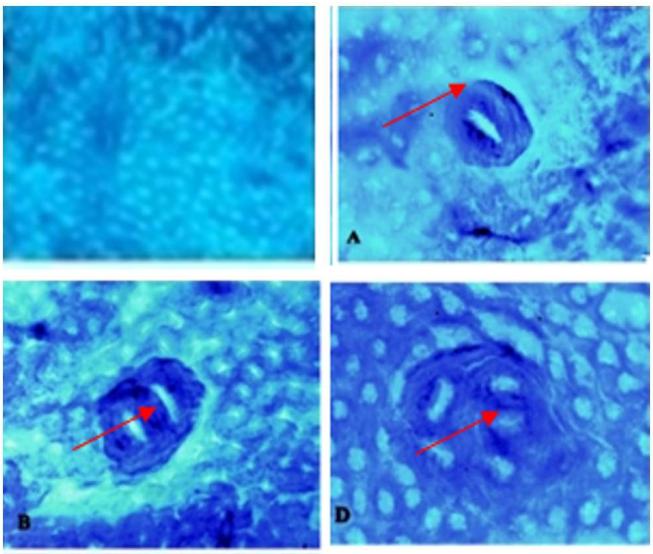

Figure 1. Aberrant crypt foci: (a) Represents normal colon, **(b)** Represents Colonic ACF with 1 Crypt, **(c)** Represents Colonic ACF with 2 crypts, **(d)** Represents Colonic ACF with ≥4 crypts.

Colonic Malondialdeyde levels

Lipid peroxidation is a consequence of oxidative stress. MDA is an index of lipid peroxidation. High MDA levels indicate high extent of lipid peroxidation in the tissue due to oxidative stress. Table 2 summarizes the data on the effect of a Ranolazine on MDA levels in colon of mice. In normal control group, average MDA value is 24.58 ± 3.314. In disease control group average MDA levels are raised to 49.326 ± 1.54 due to induction of oxidative stress by DMH. In the groups treated with Ranolazine (10 mg/kg), the average MDA levels are decreased to 37.90 ± 3.298. In the groups treated with Ranolazine (20 mg/kg), the average MDA levels are decreased to 26.62 ± 1.482, which is considered as statistically significant ($p<0.001$), when compared with normal and standard groups.

Colonic superoxide dismutase levels

Superoxide dismutase is an important antioxidant defense enzyme system present in all cells exposed to oxygen. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Oxidative stress results in depletion of SOD levels in the colonic tissue. Table 3summarizes the data on the effect of a Ranolazine on SOD levels in colon of mice. In normal control group, average SOD value is 5.12 ± 0.602. In disease control group average SOD levels are decreased to 2.13 ± 0.835 due to induction of oxidative stress by DMH. In the groups treated with Ranolazine (10 mg/kg), the average SOD levels are increased to 4.37 ±

Table 1. Effect of Ranolazine (10 mg/kg and 20 mg/kg) on DMH-induced colonic ACF formation in Swiss albino mice

Groups	Number of ACF			
	1 crypt	2 crypts	3 crypts	≥ 4 crypts
Disease control	67.34±1.570	36.9±2.670	42.3±1.05	30.66±1.570
Standard group	29.77±0.499[b]	7.90±9.90[a]	10.8±1.679[a]	19.23±0.499[b]
Ranolazine (10 mg/kg)	40.80±2.690[b]	15.78±2.570[b]	24.9±2.320[b]	19.80±2.690[b]
Ranolazine (20 mg/kg)	10.9±1.560[a]	7.0±0.750[a]	3.2±0.897[a]	4.2±1.560[a]

Data was expressed as mean ± SEM (n=4). [a]$p<0.001$,[b]$p<0.01$ compared to disease control group.

Table 2. Effect of Ranolazine (10 mg/kg and 20 mg/kg) on oxidative parameters in DMH induced colon cancer in mice

Groups	MDA (nmol/mg)	SOD (µmol/g)	Catalase (k/ml)	GSH (µmol/g)
Normal control	24.58 ± 3.314	5.12 ± 0.602	0.588 ± 0.093	4.73 ± 0.555
Disease control	49.326 ± 1.54$^{\alpha}$	2.13 ± 0.835$^{\alpha}$	0.276 ± 0.052$^{\alpha}$	2.70 ± 0.519$^{\alpha}$
Standard control	30.32 ± 2.467^{b}	4.27 ± 0.592^{a}	0.53 ± 0.146^{a}	3.79 ± 0.358$^{\gamma,b}$
Ranolazine (10 mg/kg)	37.90 ± 3.298$^{\gamma,b}$	4.37 ± 0.513^{a}	0.492 ± 0.05$^{\gamma,b}$	4.54 ± 0.394^{b}
Ranolazine (20 mg/kg)	26.62 ± 1.482^{a}	5.07 ± 0.720^{a}	0.589 ± 0.107^{a}	4.67 ± 0.39^{a}

Data was expressed as mean ± SEM (n=4). $^{a}p<0.001$,$^{\gamma}p<0.05$ compared to Normal control group, $^{a}p<0.001$,$^{b}p<0.01$ compared to Disease control group.

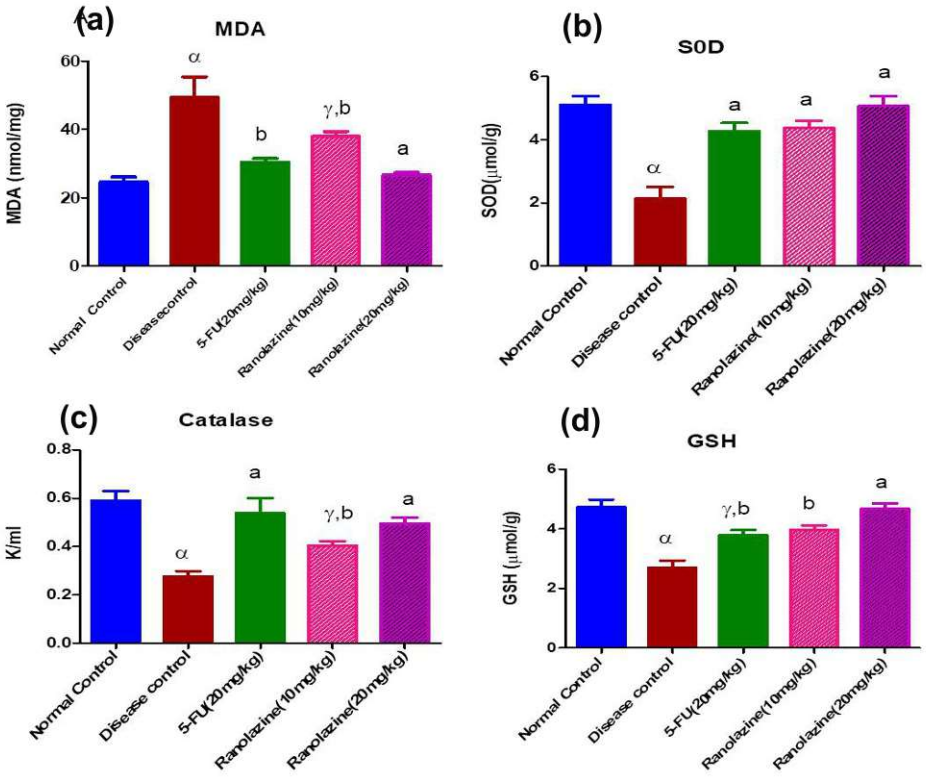

Figure 2. Modulatory effects of Ranolazine on: **(a)** MDA levels in colon **(b)** SOD levels in colon **(c)** Catalase levels in colon **(d)** GSH levels in colon

0.513. In the groups treated with Ranolazine (20 mg/kg), the average SOD levels are increased to 5.07 ± 0.7202, which is considered as statistically significant ($p<0.001$), when compared with normal and standard groups.

Colonic catalase levels

Catalase is an antioxidant enzyme system present in all living cells exposed to oxygen. Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen and thus protects the cell from oxidative damage. Oxidative stress may result in decrease in catalase levels in the colonic tissue. Table 4 summarizes the data on the effect of a Ranolazine on catalase levels in colon of mice. In normal control group, average catalase value is 0.588 ± 0.093. In disease control group average catalase levels are decreased to 0.276 ± 0.052 due to induction of oxidative stress by DMH. In the groups treated with Ranolazine (10 mg/kg), the average catalase levels are increased to 0.492 ± 0.05. In the groups treated with Ranolazine (20 mg/kg), the average catalase levels are increased to 0.589 ± 0.107, which is considered as statistically significant ($p<0.001$), when compared with normal and standard groups.

Colonic GSH levels

GSH is a free radical scavenging system, acts by preventing damage to cellular components from reactive oxygen species. Increased oxidative stress may cause depletion in GSH levels in the colonic tissue. Table 5 summarizes the data on the effect of a Ranolazine on GSH levels in colon of mice. In normal control group, average GSH value is 0.588 ± 0.093. In disease

control group average GSH levels are decreased to 2.70 ± 0.519 due to induction of oxidative stress by DMH. In the groups treated with Ranolazine (10 mg/kg), the average GSH levels are increased to 4.54 ± 0.394. In the groups treated with Ranolazine (20 mg/kg), the average GSH levels are increased to 4.67 ± 0.39, which is considered as statistically significant ($p<0.001$), when compared with normal and standard groups.

Histopathologic examinations

Histopathologic findings of colon in normal mice revealed normal mucosal (Red arrows), sub mucosal is normal (White arrows) with regular crypts with no inflammation (Figure 3a). The group treated with DMH, colonic tissue has shown inflammatory cells in the submucosal layer (white arrows) and the colonic tissue exhibited dysplasia (Figure 3b). In the group treated with standard drug, 5-fluorouracil, there is moderate inflammation in the submucosa (white arrows), and moderate dysplasia (Figure 3c). The group treated with Ranolazine 10 mg/kg, has shown moderate submucosal inflammation (white arrows) and moderate dysplasia. The group treated with Ranolazine 20 mg/kg has shown normal mucosa (red arrows) and normal submucosal layers (white arrows) with no inflammation and dysplasia (Figure 3d). Mice treated with Ranolazine 20 mg/kg exhibited normal histology as that of normal control group (Figure 3e).

Discussion

The present study demonstrated that administration of Ranolazine significantly reduced DMH induced colon cancer in mice evidenced by decreased ACF formation and restored oxidative parameters.

The earliest identifiable morphological changes in colonic mucosa in the chronology of cancer development are aberrant crypts (ACF). These aberrant crypts can be identified by their increased size, thicker epithelial lining and increased pericryptal zone. ACF formation is known as a putative indicator of colon carcinogenesis and efficacy of anti-carcinogenic effects. In fact, ACFs are considered the gold standard of colon carcinogenesis biomarkers. In agreement with previous reports, it was observed that mice treated with DMH showed significantly high number of aberrant crypt foci. Anticancer effect of Ranolazine was evidenced by decrease in total number of aberrant crypt foci per mice.

DMH is a carcinogen that undergoes metabolic activation in the liver to produce active electrophilic carbonium ion, which in turn through several process, is known to elicit oxidative stress. Reactive oxygen species, due to their higher reactivity are thus potentially toxic, mutagenic, or carcinogenic. Increased free radical activity, including enhanced lipid peroxidation is considered to play a major role in the

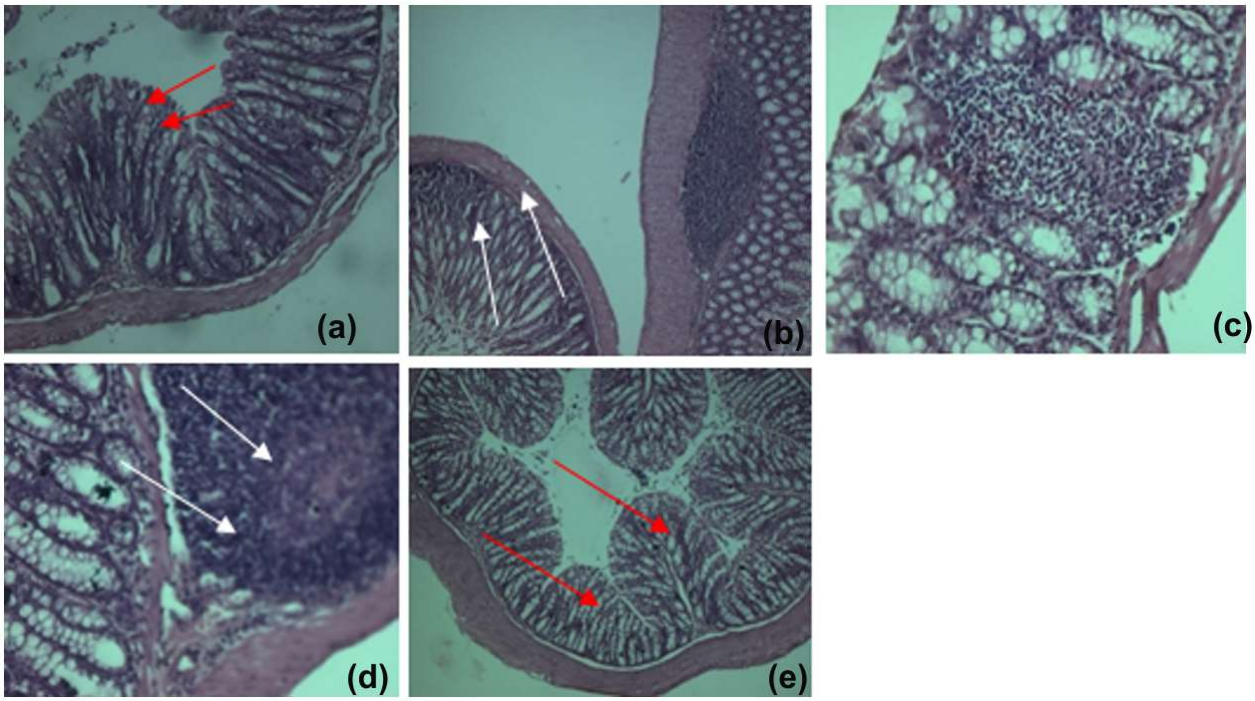

Figure 3. Histopathological observation: **(a)** In normal group the mucosa (red arrows) is normal and submucosa (white arrows) is normal, **(b)** In disease group there is submucosal inflammation (white arrows), **(c)** In standard group there is moderate submucosal inflammation (white arrow), **(d)** In Ranolazine 10 mg/kg group there is moderate inflammation (white arrow), **(e)** In Ranolazine 20 mg/kg group mucosal (red arrows) and submucosal layer (white arrows) is normal.

pathogenesis of cancer. There is considerable evidence to indicate that the severity of oxidative stress in carcinogenesis is associated with the decreased antioxidant defense.

Mice treated with DMH showed high incidents of aberrant crypt foci along with malondialdehyde (MDA) and decreased GSH, SOD and Catalase levels. Histopathalogical evaluation of colon in DMH treated mice also showed dysplasia, inflammation and focal congestion in sub-mucosa and muscularis layers. The treatment with Ranolazine reversed all the above effects and restored to the normal level.

Histopathological studies revealed inflammation, severe dysplasia and focal congestion in muscularis mucosa and sub-mucosal layers in mice treated with only DMH. Histological evaluation of colonic tissues of mice treated with Ranolazine showed suppressed inflammatory responses in the colon by decreasing the intense infiltration of the inflammatory cells in the mucosal and sub mucosal layers and also exhibited mild dysplasia with very mild focal congestion.

Conclusion

In conclusion, the present study suggests that treatment with Ranolazine significantly decreased the DMH induced colon cancer probably due to deregulations of cancer cell progression. Thus these findings may open novel prospective in cancer chemotherapy.

Conflicts of interest: Not declared

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