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Research Article

Evaluation of Oxidative Stress in Azoxymethane-induced Colon Cancerous Fischer Rats

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Abstract

Objective: to study the redox status of normal colon and aberrant crypts formed in azoxymethane induced colon cancerous fischer rats.

Methods A total of 16 five-week-old male Fisher 344 rats (*Rattus norvegicus*), weighing approximately 90–100 grams were housed individually in plastic cages with wood-chip bedding. The animals were acclimatized for 1 week and fed with an American Institute of Nutrition (AIN-93G) diet *ad libitum*. Their protein oxidation, DNA damage, lipid peroxidation and antioxidants, glutathione (GSH), and antioxidative enzymes in serum were detected.

Results The levels of protein oxidation Sand lipid peroxidation were significantly higher in the study group than in the control group ($P<0.01$). However, the mean serum level of MDA and conjugated diene was lower in the study group than in the control group ($P<0.01$). The activity of antioxidative enzymes was significantly decreased in the study group compared to control group ($P<0.01$).

Conclusion Colorectal cancer is associated with oxidative stress, and assessment of oxidative stress and given antioxidants is important for the treatment and prevention of colorectal cancer.

Keywords: colorectal cancer; azoxymethane, fischer rats; oxidative stress; lipid peroxidation; protein oxidation

Introduction

Zinc is a trace element necessary for a variety of physiological and biochemical functions, including the integrity of the intestinal barrier and gut-associated immune function, reduction of oxidative stress and inhibition of apoptosis (Skrajnowska & Bobrowska-Korczak, 2019; Gombart et al., 2020). Zinc is directly involved in crypt cell proliferation (Ohashi & Fukada, 2019). Recently, the mechanism of intestinal absorption of zinc was partially elucidated (Maares & Haase, 2020).Because CRC treatment plan needs to consider nutritional responses towards anti-cancer drugs based on their biological and genetic characteristics, a possible association with zinc in cancer treatment also requires attention. Therefore, we analyze the physiological and biological implication of Zn on CRC to provide screening, treatment, and prevention strategies.

Significant alterations in Zn (II) levels in tissues have previously been reported in patients with various forms of cancer. Moreover, low plasma Zn (II) levels have been observed in patients with cancer of the colon, bronchus or digestive system (Michalczyk & Cymbaluk-Płoska, 2020). There is a clear association between zinc and risk of CRC. Intervention

plan in this type of cancer needs to consider nutritional responses towards anti-cancer drugs based on their biological and genetic characteristics; furthermore, a possible association with zinc in cancer treatment also requires attention. The analysis of Zn biomarkers levels could provide new biological insights applied in prevention, molecular diagnosis, prognosis and treatment of CRC (Mehta et al., 2010; Rahman et al., 2019).

The mechanism involved in chemoprevention of colorectal cancer is not clear; hence this study will form a basis to understand the role of zinc in preventing colorectal cancer. Various studies have contributed to the understanding of dietary supplementation of iron and zinc as chemo-preventive agents after formation of pre-neoplastic lesions (Dhawan & Chadha, 2010), but have not elucidated the role of endogenous trace minerals in the formation of pre-neoplastic lesions induced by azoxymethane.

Zinc has also been a part of certain proteins structures (~3000 proteins/ 10% of the human proteome) (Andreini *et al.,* 2006; Sharma et al., 2018). Some zinc requiring enzymes can be directly related to a host defense against initiation and progression of cancer, including colon cancer (Prasad, 2008; Prasad, 2014; Fukada et al., 2011; Fukada, 2015). In fact,

some experimental studies have showed a protective action of zinc sulphate supplementation against rat colon carcinogenesis induced by the carcinogen 1, 2-dimethylhydrazine (DMH) (Dani et al., 2007). A reduction in tissue zinc levels and antioxidant enzymes have been detected during progression of preneoplastic lesions to malignant tumors in the colon of male rats (Dani et al., 2007). In addition, zinc deficiency advanced the development of adenomatous polyps to carcinoma in situ but delayed the progression of carcinoma in situ to invasive adeno-carcinomas induced by DMH in male mice (Tanaka, 2009). It is known that excessive ROS are formed in chronic diseases of the gastrointestinal tract, but the precise mechanism underlying oxidative stress in cancer cells and molecular pathogenesis of CRC remains to be understood. The main objective of the present study is to study the redox status of normal colon and aberrant crypts.
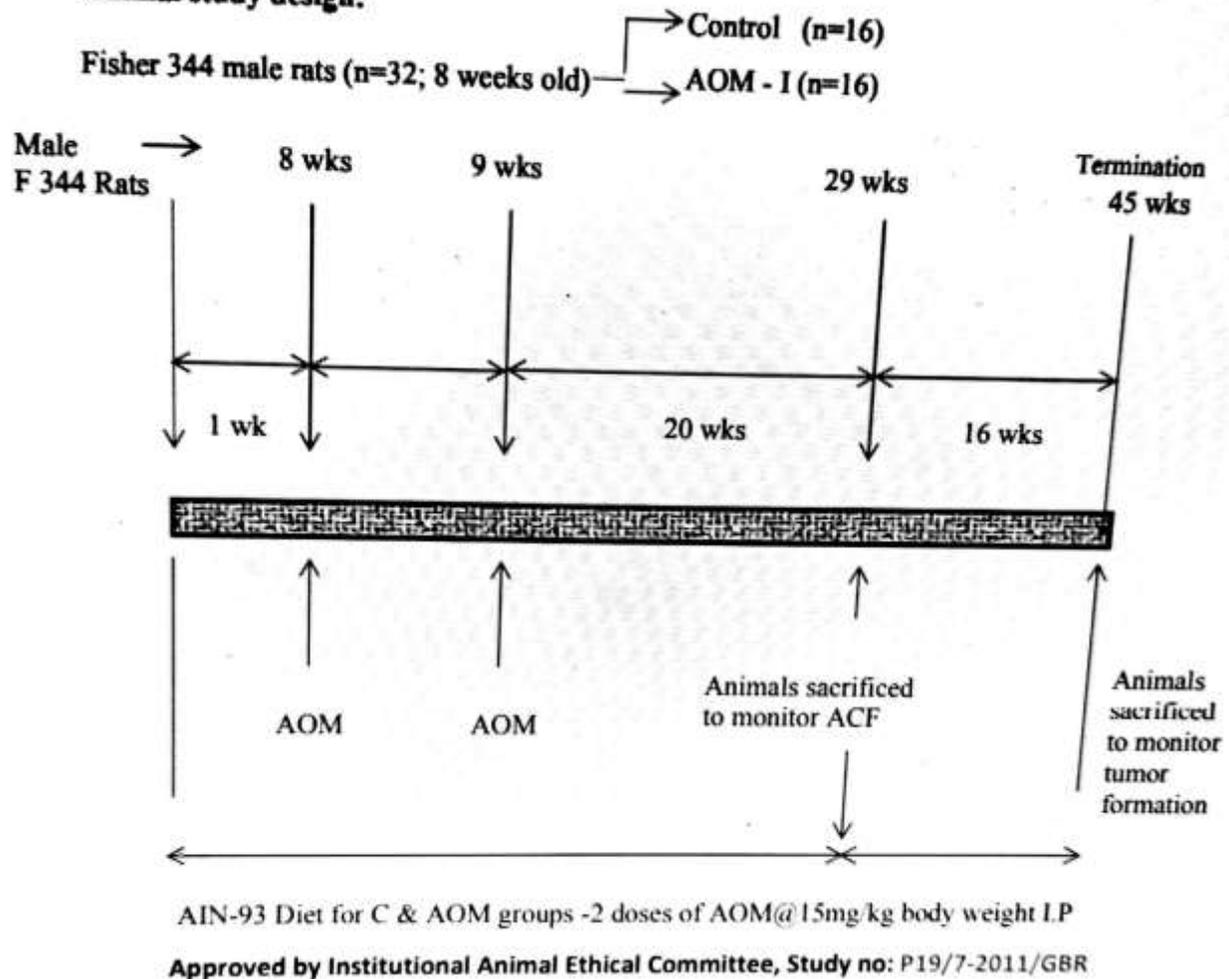
Materials and methods

Chemicals and reagents

AOM, 10% (v/v) neutral buffered formalin were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Diet and animals

This study was performed according to the guidelines approved by the Institutional Animal Care and Ethics Committee (IACEC) of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad. A total of 16 five-week-old male Fisher 344 rats (*Rattus norvegicus*), weighing approximately 90–100 grams were housed individually in plastic cages with wood-chip bedding. The animals were acclimatized for 1 week and fed with an American Institute of Nutrition (AIN-93G) diet *ad libitum* (Satya Prasad et al., 2018). They were housed in a well-ventilated room at approximately 25 to 27°C, 50 ± 10% relative humidity, and a 12-hour light/dark cycle. Hygienic conditions were maintained by weekly changes of woodchip beds. The rats were randomly assigned to control (n = 8) or AOM (n=8) groups. AOM group received azoxymethane intraperitoneally (15 mg/kg body weight) diluted in 0.9% (v/v) saline, once weekly for two successive weeks. Control group received an equal volume of saline and served as the vehicle control. After 20 weeks of initiation, animals were sacrificed to study the formation of aberrant crypt foci in colon, in addition to tissue zinc measurements (Kavitha et al., 2021). Food consumption was recorded daily and the body weight was recorded weekly throughout the study. Aliquots of blood were obtained with EDTA as anticoagulant to measure plasma zinc. The colonic enterocytes were removed, washed with ice-cold saline solution (0.9% NaCl, wt/vol), weighed, were split into three random fractions, snap frozen in liquid nitrogen, and stored immediately at −20°C until further analysis.

Animal study design:

Fisher 344 male rats (n=32; 8 weeks old) → Control (n=16) / AOM - I (n=16)

AIN-93 Diet for C & AOM groups -2 doses of AOM@15mg/kg body weight I.P

Approved by Institutional Animal Ethical Committee, Study no: P19/7-2011/GBR

Zn Determination in the Diets and Liver

Samples of diets and tissue (1 g of DW) were mineralized following the method described previously. Zinc concentrations in the different diets were determined by inductively coupled plasma mass spectrometry (Shimadzu AA7000).

Abberent Crypt Foci (ACF) analysis and tumor assessment:

ACF analysis was performed according to Bird (1987), after sixteen weeks of AOM administration. The colons were longitudinally opened, rinsed with saline and fixed flat between two sheets of filter paper in 10% buffered formalin for at least 24 h. The colons were then cut into 2 cm segments, and stained with 0.2% methylene blue in Krebs-Ringer solution for 10 min, and were then placed mucosal side up on a microscope slide and counted through a light microscope at 400 x magnification. Crypts with increased size, increased separation from lamina to basal surface, and appearance of pericryptal zone were distinguished as aberrant crypts. Number of crypts in each focus determined the crypt multiplicity.. Number of crypts in each focus determined the crypt multiplicity. For tumor assessment at the end of 36 weeks, colon tissue was removed, dissected longitudinally, flushed with phosphate-buffered saline (PBS), fixed in 10% (v/v) neutral buffered formalin prior to staining with hematoxylin and eosin (H&E). The tumor incidence was described as the percentage of total animals with adenoma/adenocarcinoma while tumor multiplicity was defined as the average number of tumors per tumor-bearing rat. The number and size of the colon tumors present was also recorded.

Blood Sampling

Fasting venous blood samples collected from each subject were placed on ice and centrifuged at 3500 rpm for 15 min at 4° C. The serum was stored at -20° C

Measurement of Antioxidants

Activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase

(CAT) in serum and total serum glutathione were detected as previously described (Beutler et al., 1963; Paglia and Valentine, 1967; Aebi, 1984; Sun et al., 1988).

Lipid Peroxidation To 1 ml of 12,000 g supernatant containing 400 μg protein (if necessary the volume was made up to 1 ml with TrisHCl buffer) equal volume of 10% TCA was added followed by the addition of 1 ml of 0.67% thiobarbituric acid. This was heated for 10 mins in a boiling water bath in a tightly stoppered tube. After cooling, the tubes were centrifuged to separate the precipitated proteins. The absorbance of the pink color supernatant was read at 532 nm. MDA standards were also run simultaneously (0.1-5 nmoles). The values are expressed as nmoles MDA/mg protein.

Protein Oxidation (Protein Carbonyl Levels)

The method adopted was according to Reznick and Packer (1994) One ml of intestinal cytosol equivalent to 1mg protein was added to 4 ml of DNPH solution which was vortexed and incubated for 1h at room temperature in dark. After the addition of 5 ml of 20% TCA, tubes were left in ice for 10 mins. This was centrifuged at 3000 rpm for 10 min and collected the protein precipitate. The protein pellet was washed thrice with 4ml of ethanol-ethyl acetate solution to remove free DNPH and lipid contaminants. The final protein precipitate was dissolved in 2 ml of 6M guanidine hydrochloride solution and left for 10 min at 37℃ with vortex mixing. Insoluble materials at this stage were removed bya n additional centrifugation. A spectral scan was performed in the supernatant between 350-390 nm. A peak corresponding to protein hydrazone was observed at 372 nm.

Determination of protein content

Because 10-15% of proteins are lost in various washing steps, the protein in the final 6M guanidine hydrochloride solution was quantitated by reading the absorption at 280 nm. The amount of protein was calculated from a BSA standard curve (0.25-2 mg/ml) which was also dissolved in 6 M guanidine hydrochloride.

Calculation

The molar extinction coefficient of dinitrophenyl hydrazine $\varepsilon=22,000 = 22000/10^6$/nmole/ml is used to calculate the concentration of carbonyls in a given sample.

Reduced glutathione

Reduced glutathione was estimated according to the method of Hissin and Hilf (1976).
To 0.2ml of 12,000 g supernatant, an equal volume of 25% HPO$_3$ was added and the volume made up to 1.2 ml with buffer. This was centrifuged at 3000 rpm for 5 min and 0.025 ml of the supernatant was taken and added to 2.375 ml of the buffer. This was followed by the addition of 0.1 ml of OPT solution. The solution was thoroughly mixed and left at room temperature for 15 min. The solution was then transferred to a quartz cuvette and fluorescence measured in Perkin-Elmer LS-3B fluorescence spectrometer with excitation at 350 nm and emission at 420 nm.
GSH levels are expressed as μmol GSH/mg protein.

Glutathione peroxidise (EC 1.11.1.9)

Gpx in the cytosolic fraction of the intestine was determined by the coupled assay procedure of Paglia and Valentine (1967). Preincubated the reaction mixture (EDTA, 1mM; Sodium azide, 1mM; NADPH, 0.2 mM; GSH, 1 mM; Glutathione reductase, and cytosolic preparation of colon from cancerous rats in phosphate buffer, pH 7.2) for 2mins at 25^0c and initiated the reaction by the addition of 0.1ml of cumene hydroperoxide solution. Absorbance at 340nm was recorded for 1min using Cary 100 Bio UV-Vis spectrophotometer. The activity of Gpx was then calculated as the amount of NADPH oxidized/min/mg protein.Enzyme activity is expressed as μmoles of NADPH oxidized/mg protein/min.

Cu, Zn- Superoxide Dismutase (EC 1.15.1.1)

SOD was estimated according to the procedure of Marklund and Marklund (1974).
The reaction was started by the addition of pyrogallol and the increase in absorbance at 420 nm was noted in a Cary UV Spectrophotometer. Simultaneously, a blank was also run without the enzyme.
One unit of SOD activity is the amount of the enzyme that inhibits the rate of auto-oxidation of pyrogallol by 50%. Activity of SOD is expressed as Units/min/mg protein.

Catalase (EC 1.11.1.6)

The activity of catalase in 12,000 g supernatant was measured according to the method of Aebi (1983). To 0.1 ml (125 μg protein) of the 12,000 g supernatant, 30 μl absolute ethanol was added to a final concentration of 0.17 M. Samples were incubated for 30 min in an ice water bath. (This procedure decomposes the complex II, which is an inactive complex of catalase). After 30 min, 10% Triton X-100 was added to a final concentration of 1% and vortexed immediately. To this was added, 1.9 ml of phosphate buffer and transferred to a quartz cuvette. The reaction was started by the addition of 1 ml of H$_2$O$_2$ solution. The decrease in absorbance at 240 nm was followed for 1 min (Cary 100 Bio UV-Vis Spectrophotometer). An enzyme blank and substrate blank was also run simultaneously.

The activity of catalase is calculated from the change in absorbance at 240 nm. One unit of catalase activity $K= (2.303/t)$ $(\log A_1/A_2)$ where K is the rate constant, t is time in min, A_1 is the absorbance at time 0 and A_2 is the absorbance at 1 min. Specific activity is expressed as units/min/mg protein.

Statistical analysis

All the results are expressed as mean ± SE. Statistical analysis was done by one way ANOVA sing statistical packages for Social Sciences [Version 10.0, Chicago, USA] wherever it is necessary. The difference between the groups were considered to be significant if $p \leq 0.05$. The difference in food intake, bodyweights, hemoglobin and serum protein levels were analyzed by repeated measures ANOVA.

Results:

ACF analysis and tumor incidence in AOM treated animals

All the rats treated with AOM developed ACF, whereas no evidence of ACF could be detected in the colons of control animals. Crypt multiplicity analysis of ACF indicated that all segments of the large intestine showed similar number and incidence of crypts per focus. Normal mucosa showed crypts arranged in parallel with round nuclei and mucin stained blue (Figure 1A), whereas hyperplastic aberrant crypt foci showed mucin depletion and elongated nuclei (Figure 1B). Representative images of normal and aberrant crypt stained with methylene blue, aberrant crypt foci, observed in AOM-administered animals is shown in Figure 1. The mean number of ACF per entire colon in the AOM administered animals was 155±14.
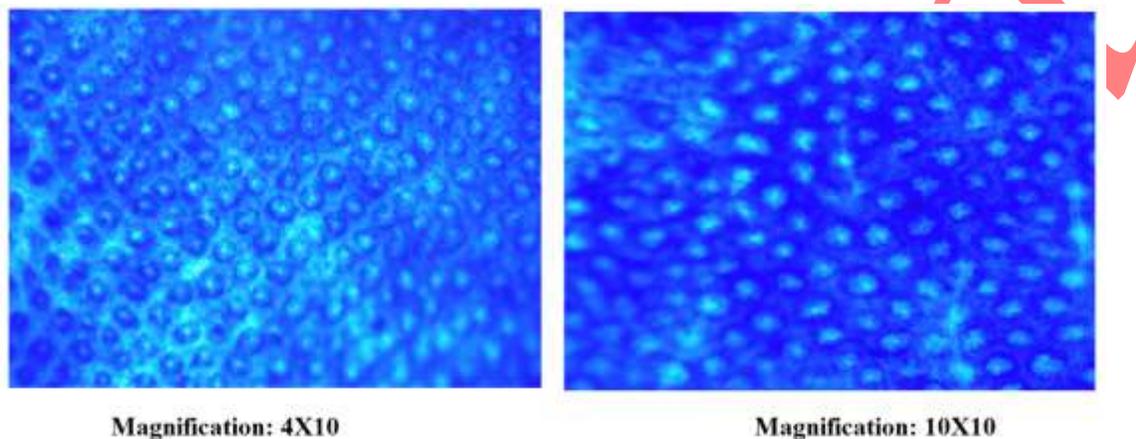

Magnification: 4X10   Magnification: 10X10

Figure 1A-*Control Animals – Normal Mucosa – 20 weeks*

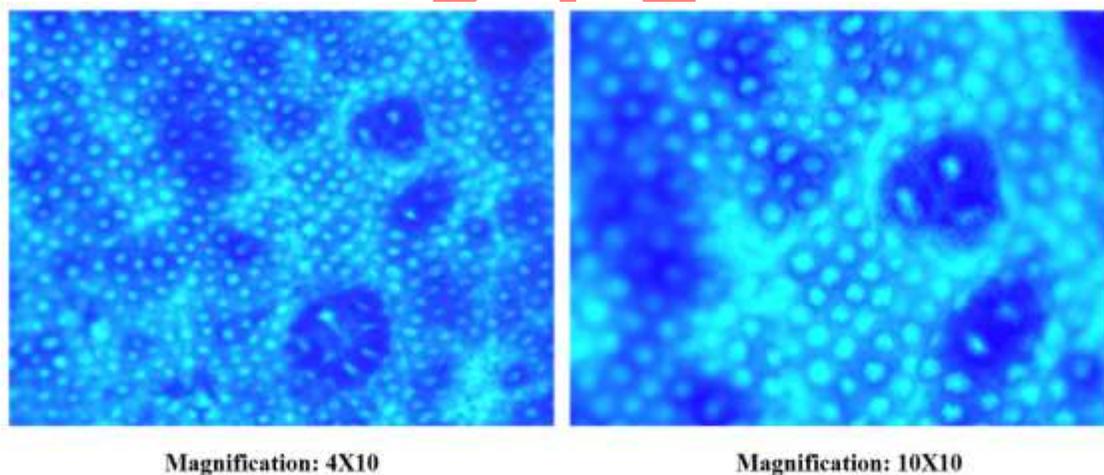Magnification: 4X10   Magnification: 10X10

Figure 1B-*Azoxymethane treated Animals – Aberrant crypt foci*

All the rats (n=8) which were treated with AOM, developed ACF

From the above figures it is clearly observed that after 20 weeks, all the rats injected with AOM developed ACF, whereas the controlled animals were normal. Crypt multiplicity analysis of ACF indicated that all segments of the large intestine showed similar number and incidence of crypts per focus. Normal mucosa arranged in parallel with round nuclei and mucin stained blue under 4X and 10X magnifications (Figure 1A), Hyperplastic aberrant crypt foci showed mucin depletion and elongated nuclei under 4X and 10X magnifications (Figure 1B).
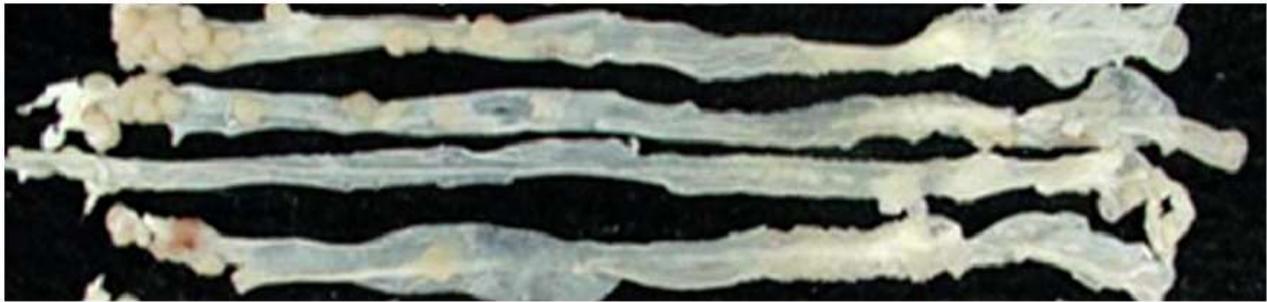
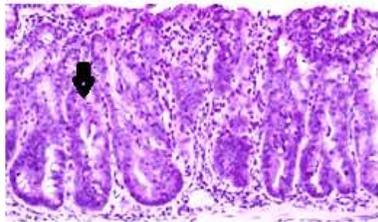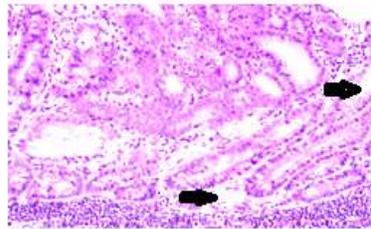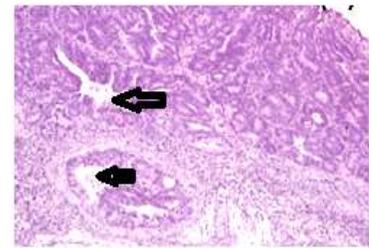Figure 2A: Colonic tumours developed in Fisher 344 rats -36 weeks

Figure 2B: (i) Dysplastic crypts   (ii) Adenomas   (iii) Adenocarcinomas

Figure 2A *shows the tumor development in Fisher 344 rats treated with AOM for 36 weeks. Many tumors were observed along the length of the colon.* **Fig 2B** *shows the histopathological analysis of cross sections of tumor tissue. Gradual development of tumor from dysplastic crypts (Figure 2Bi) to adenomas (Figure 2Bii) to adenocarcinomas (Figure 2Biii) can be seen in the tissue sections.*

Diet zinc was maintained at 35 mg of Zn/kg diet.

Plasma zinc levels were decreased in AOM treated rats

Plasma zinc concentrations in control and AOM-administered rats at the end of 20 weeks were 110±8.5 and 78± 6.2 µg/dL respectively. These results clearly indicate altered systemic zinc concentrations in AOM administered animals.

Antioxidant enzyme levels were significantly altered in AOM treated rats

In precancerous both Mn-SOD and Cu-Zn SOD show no change when compared to control. In tumour tissue the Mn-SOD shows no significant change when compared to precancerous tissue but a significant increase is observed when compared to control. The Cu- Zn SOD of tumour tissue shows significant increase when compared to control and precancerous (figure 3).
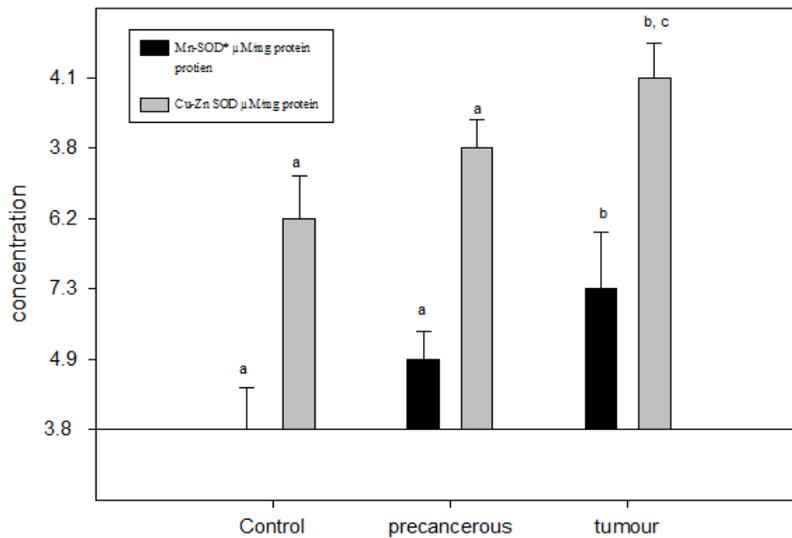
Superoxide dismutase (SOD)

Figure-3 *Antioxidant status of two isoforms of Superoxide dismutase (SOD) – Mn-SOD and Cu-Zn SOD in precancerous and cancerous lesions.*

When compared to control there is significant increase in the glutathione peroxidase of the precancerous and cancerous tissue. The increase is markedly observed in the cancerous tissue when compared to the precancerous tissue (figure 4). There is no difference between the glutathione S- transferase of control and precancerous tissue but there is significant increase in the glutathione S- transferase of cancer tissue when compared to precancerous (figure 5).

When compared to control there is significant increase in the MDA (malondialdehyde) of the precancerous and cancerous tissue. The increase

is markedly observed in the cancerous tissue when compared to the precancerous tissue (figure 6). When compared to control there is significant increase in the catalase of the precancerous and cancerous tissue. The increase is markedly observed in the cancerous tissue when compared to the precancerous tissue (figure 7). There is gradual decrease in the glutathione of the precancerous and cancerous tissue. The decrease is markedly observed in the cancerous tissue when compared to control (figure 8).
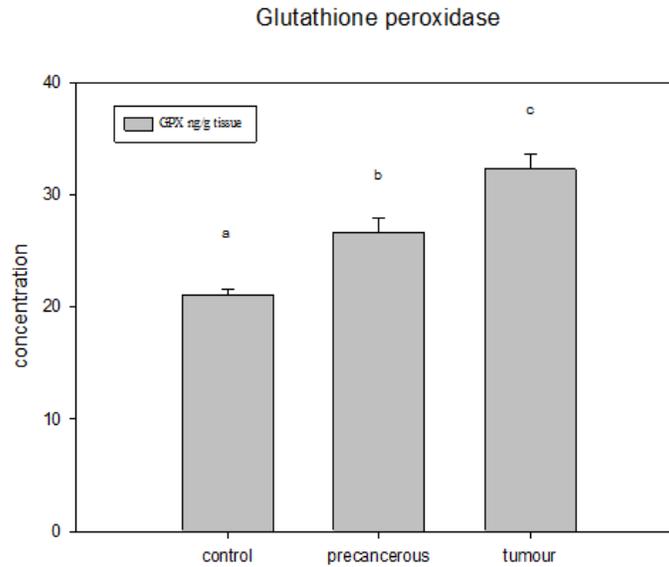

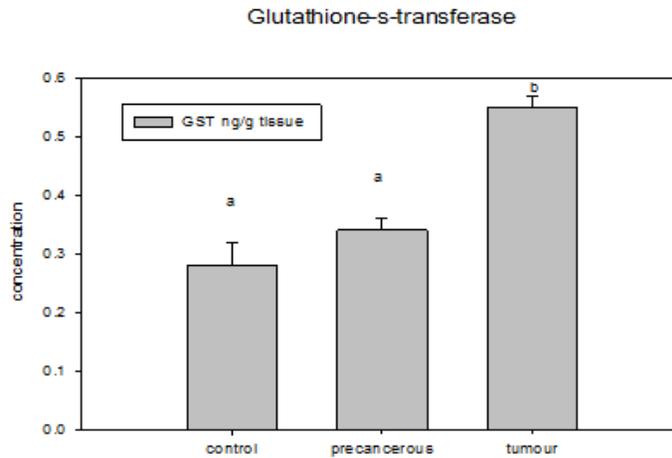**Figure-4** *Antioxidant status of glutathione peroxidase in precancerous and cancerous lesions.*

Figure-5 *Antioxidant status of Glutathione S-transferase*

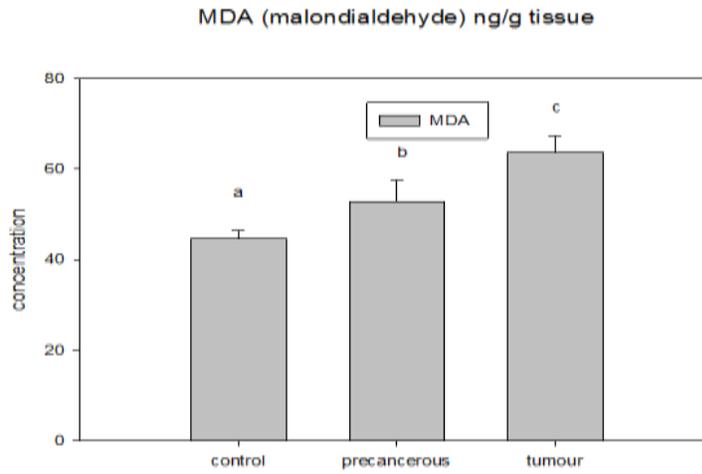## MDA (malondialdehyde) ng/g tissue

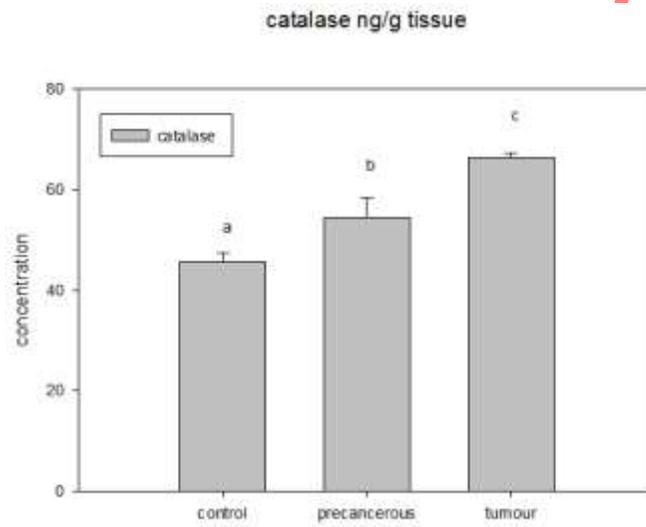**Figure-6** *Antioxidant status of MDA (malondialdehyde)*

catalase ng/g tissue

Figure-7 *Antioxidant status of Catalase*
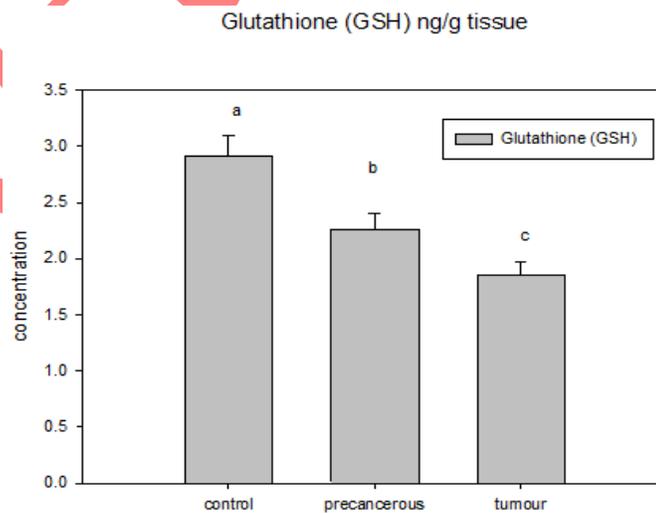
Glutathione (GSH) ng/g tissue

Figure-8 *Antioxidant status of Glutathione (GSH)*

Discussion

Zinc homeostasis is primarily regulated through alterations in intestinal Zn transport whereas zinc deficiency increases dietary zinc absorption. Two families of Zn transporters have been identified that participate in Zn homeostasis regulation. The Zip family imports Zn into the cytoplasm, either across the plasma membrane or out of intracellular vesicles. Zip4 is presumed to be the primary regulator of intestinal Zn import, because mutations in the gene that encodes Zip4 (SLC39A4) are responsible for acrodermatitis enteropathica, a rare genetic disorder in humans that results in severe Zn deficiency from impaired Zn absorption.

Metallothioneins are proteins involved in heavy metal homeostasis and detoxification, providing protection against hydroxyl free radicals. The bioavailability of zinc in acute phase response to tissue injury and inflammation is controlled by metallotionein (MT1G).

Recently Liu et al.(2017) showed metallothionein overexpression in hyperplastic epithelium of the oesophagus in zinc-deficient rats. In contrast, other studies demonstrated that zinc deficiency in growing and adult rats lead to decreased expression of metallothioneins.

Aberrant crypt foci (ACF), which are recognized as early appearing preoplastic lesions, develop in experimentally induced colon carcinogenesis in laboratory rodents. ACF were first described by Bird (1987) and are defined as crypts that have altered luminal openings; exhibit thickened epithelia; and are larger than adjacent normal crypts. It is known that the crypts of ACF have increased proliferative activity and some ACF reveal histological dysplasia. Pretlow et al. (1993) showed the presence of such lesions in the colonic mucosa of patients with colon cancer and suggest that aberrant crypts are precursor lesions from which adenomas and carcinomas develop in the colon. Recently, during the early stages of AOM-induced colon carcinogenesis, catenin-accumulated crypts were identified in the colonic mucosa and are considered as early-appearing preneoplastic lesions (Yamada and Mori, 2003). Therefore, ACF are regarded as putative preneoplastic lesions for colon cancer and are used as biomarkers to evaluate potential chemo preventive agents against colon carcinogenesis (Reddy et al., 1996).

Super oxide dismutases (SOD) are metalloenzymes that play a vital role in the protection of aerobic cells against oxygen toxicity and cytosolic CuZnSOD has been shown to contain both copper and zinc atoms (Justin et al., 2017).

It has been documented that significant oxidative stress, caused by free radicals, occurs in carcinoma of the intestinal mucosa. In particular, the super oxide radical ion has been postulated as the possible cause of cancer (Phanindra et al., 2015).

AOM-induced histological differentiation in the colon was directly related to a decrease in concentration of plasma zinc, tissue zinc and reduced zinc-related CuZnSOD enzyme activity. These might be successively identified with the biochemical occasions in the internal organ such as large intestine in AOM treated rodents. In this study, the trend towards a gradual decrease in zinc concentration in the malignant tissue is in agreement with the results observed in cancer patients by Pamela et al., (2010).

In conclusion, this study indicates that there is a change in tissue zinc concentration and CuZnSOD activity, which occurs alongside the changes in the mucosa during precancerous transformation. The most significant change was observed in the large intestine at 9 months after AOM induction, and the histology shows a progression of precancerous lesion in the colon with 2 out of 6 rats developing an obvious precancerous lesion. The animal model described in the present study is suitable for further studies on many aspects of tumor development in the colon and gastrointestinal tract. The exact mechanisms by which tissue zinc and its related enzyme CuZnSOD decrease in colon carcinomas remain to be elucidated.

Other enzymatic antioxidant defense systems include SOD, CAT, GSH-Px, GSH. These antioxidants play their important role in normal metabolism and healthy state of the animal body (Zhou et al., 2002) and are altered in cancer subjects (Ozdemirler Erata et al., 2005; Skrzydlewska et al., 2005; Rainis et al., 2007; Devasena et al., 2007). They are found in varying locations of cells and play an important role in scavenging oxygen free radicals (Muse et al., 1994). Decrease in antioxidants may break the balance between pro- and anti-oxidants, leading to cellular damage and ultimately malignant transformation. In our study, the activities of SOD, GSH-Px, CAT were significantly decreased in CRC patients, showing that the presence of oxidative stress in these patients supports the hypothesis of radical-mediated injury in CRC rats. Our findings are in agreement with the reported data (Ozdemirler Erata et al., 2005; Devasena et al., 2007). However, there are also conflicting findings (Skrzydlewska et al., 2005; Rainis et al., 2007). In conclusion, the level of oxidative stress is increased in CRC patients and oxidative stress may play an important role in the development of CRC and antioxidants might be a treatment modality of choice.

Conflict of Interests

The authors declare that there are no conflict of interests.

Data Availability Statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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