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Original Article

ROLE OF ANTIOXIDANT AND MYELOPEROXIDASE LEVELS IN 7, 12-DIMETHYLBENZ [A] ANTHRACENE INDUCED EXPERIMENTAL RAT MODEL: EVIDENCE FOR OXIDATIVE DAMAGE IN ACTIVE ULCERATIVE COLITIS.

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ABSTRACT

Objective: Ulcerative colitis known as inflammatory bowel disease (IBD) of unknown etiology. We examined the antioxidant and myeloperoxidase status in a murine model of 7, 12-dimethylbenz [a] anthracene induced colitis to elucidate the exact mechanism behind the inflammation.

Methods: Male Wistar rats were exposed to ulcerative colitis using various concentration of DMBA (7,12-Dimethylbenz[A]anthracene) were periodically analysed on 4th, 8th, 12th, 24th and 32nd week from the date of induction. To determine the disease activity index changes in body weight, food consumption, the presence of gross blood in stool and consistency of feces and diarrhea were observed. Macroscopic characters were elucidated based on clinical features of the colon and rectum using scoring pattern. Tissue inflammation status was noted through myeloperoxidase (MPO) assay. The antioxidant status in tissue samples was analysed by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and total reduced glutathione (GSH).

Results: Gavage intubation of DMBA induced colitis showed significant changes from 4th week and severity on 32nd week. The body weight was gradually reduced. Macroscopic scoring showed severe scoring pattern the inflammation was significantly heavier by week 4; and by the end of 32 w, inflammation in rats was double that of the controls, tissue myeloperoxidase (MPO) activity showed the steady increase of neutrophil infiltration and inflammation rate every week. A significant change was noted in tissue antioxidant status and it showed the oxidation level. Statistically, significant change was recorded from 4th week till 32nd week.

Conclusion: The conventional biochemical changes in colitis induced animal model revealed the association between the oxidative stress and ulcerative colitis.

Keywords: Colitis, 7, 12-Dimethylbenz [A]anthracene, Macroscopic scoring, Gavage intubation and scoring pattern

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INTRODUCTION

Ulcerative colitis is an inflammatory bowel disease (IBD) is an idiopathic disease characterized by mucosal inflammation of the gastrointestinal tract. The etiology of the disease has been extensively studies during the last several decades; however, causative factors in disease pathology are not yet fully understood [1, 2]. The primary symptoms of ulcerative colitis are diarrhea, abdominal pain, and urgency of defecate [3]. The inflammatory bowel diseases are becoming more common in Asia, but epidemiologic data are lacking [4]. A number of population-based studies in IBD have been published from Europe, North America and Australia [5, 6]. Even within Asia incidence and prevalence rates of IBD vary according to geography and ethnic groups [7]. The highest rates have been reported in India (particularly ulcerative colitis), Japan, Middle East, and overall rising trends of IBD are seen in East Asia [8]. Oxidative stress is a potential etiological and/or triggering factor for inflammatory bowel disease because the detrimental effects of reactive oxygen molecules (ROM) have been well established in the inflammation process [9]. One of the most important categories of damage contributing to inflammation is that caused by oxidative stress, as a result of the production of 'free radicals'. The free radicals also play a very important role in degenerative, neurodegenerative disorders, atherosclerosis, diabetes and inflammatory bowel disease [10, 11].

A molecule with one or more unpaired electron in its outer shell is called a free radical. Free radicals are able to attack numerous biological substances, including lipid membranes, proteins and DNA. They exert some detrimental effects, including lipid peroxidation of cell membranes, alteration of lipid-protein interactions, enzyme inactivation and DNA breakage [12]. The exogenous sources of free radicals include tobacco smoke, ionizing radiation, pollutants, organic solvents and pesticides [13]. Free radical is a tiny but highly reactive molecule and likely to damage the first thing it meets inside the cell. This might be a protein, membrane lipids, or a segment of DNA. The vast amount of damage is done daily to DNA by free radicals, estimated at 10,000 hits per cell per day. ROS are created as part of normal cellular metabolism and defense systems. At low levels, they play an important role in regulation of cellular growth, differentiation, proliferation and apoptosis [14]. They perform the roles by reversibly affecting different receptors, genes, ion channels, enzymes, proteins and nuclear transcription factors [15, 16]. A disturbance of the balance between formation of active oxygen metabolites and the rate at which they are scavenged by enzymic and non-enzymic antioxidants is referred to as oxidative stress [17]. Oxidative stress has been implicated in the etiology of several (>100) of human diseases and in the process of inflammatory bowel disease [11].

Antioxidants are substances that neutralize free radicals or their actions [18]. Generally, cells possess enzyme systems as superoxide dismutase (SOD), catalase (CAT), and non-enzyme defence system as, reduced glutathione (GSH), for both preventing the production of free radicals and repairing oxidative damage [19]. Oxidative stress has been implicated in the etiology of several of human disease. The present study was aimed to determine the relationship between the oxidative stress and ulcerative colitis.

MATERIALS AND METHODS

Chemical

712-Dimethylbenz (a) anthracene (DMBA) was purchased from Sigma-Aldrich chemicals. It was maintained at room temperature till

use and was dissolved in olive oil. The gavage tube was purchased in the market. All other chemicals and solvents used were of analytical grade [20].

Animals

Male Wistar rats (170-200 g) were obtained from the animal house of Mannuthy veterinary college, Thrissur, kerala. Animals were maintained under standard conditions (12h lighr/dark cycle; 25±3 °C, 45-65% humidity) and had free access to standard rat feed and water *ad libitum.* All the animals were acclimatised to laboratory conditions for a week before the commencement of the experiment. Animal studies were performed according to the prescribed guidelines of the committee for the purpose of control and supervision of experiments on animals (659/02/a/CPCSEA), Government of India, India.

Induction of colitis

Animals were divided into two groups of three animals each. Group, I served as normal control and received saline. Group II served as an induced group in that animals were sub-grouped as A, B. Group IIA of three rats were induced with 10 mg of DMBA/kg b.w. and Group IIB received 20 mg of DMBA/kg body weight. Chemicals were induced through gavage tube. The animals were anaesthetized and sacrificed by cervical dislocation on 4th, 8th, 12th, 24th, and 32nd week from the date of induced. Tissue's colon and rectum were exercised and washed in 0.9% saline and processed for experimental procedures.

Evaluation of the disease activity index

Colitis was determined and quantified with a clinical score over a period of 32 w after DMBA induction. Parameters screened daily were body weight, physical appearance, food consumption, observation of gross blood in stool and fecal inconsistency and diarrhea.

Macroscopic characters of severity of colitis

The severity of colitis in rats was macroscopically assessed based on the loss of body weight, the scoring of stool consistency and the presence of fecal occult blood as reported earlier [20]. In each animal, the distal 10 cm portion of the colon and rectum was removed and cut longitudinally, slightly cleaned in physiological saline to remove faecal residues and weighted. Macroscopic inflammation scores were assigned based on clinical features of the colon and rectum using following scoring patterns. No visible change was counted as 0 point, hyperemia at sites as 1 point, lesions having diameter 1 mm or less counted as 2 points, lesions having diameter 2 mm or less (<5, 5-10 and>10) as 3,4 and 5 points, respectively and lesions having diameter more than 2 mm (<5, 5-10,>10) counted as 6,7 and 8 points [21].

Myeloperoxidase (MPO) assay

The tissue homogenate was centrifuged at 800 X g for 30 min at 4 °C. The supernatant was discarded. 10 ml of ice-cold 50 mmol potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyl-trimethyl-ammonium bromide and 10 mmol EDTA was then added to the pellet. It was then subjected to one cycle of freezing, thawing and brief period (15 s) of sonication. After sonication, the solution was centrifuged at 13,100 X g for 20 min. The MPO activity was measured spectrophotometrically [23]. 0.1 ml of supernatant was combined with 2.9 ml of 50 mmol phosphate buffer containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% hydrogen peroxide. This change in absorbance was measured at 460 nm. One unit of MPO activity is defined as the change in absorbance per min by 1.0 at room temperature, in the final reaction.

Assay of antioxidants in tissue samples

Determination of superoxide dismutase (SOD) activity

1.4 ml aliquot of the reaction mixture in a test tube containing 1.1 ml of 50 mmol phosphate buffer, 75 µl of 20 mmol L-methionine, 40 µl triton of 1% X-100, 75 µl of 10 mmol hydroxylamine hydrochloride and 100 µl of 50µM EDTA. To the tube, 100 µl of the sample was added followed by pre-incubation at 37 ^{O}C for 5 min. 80 µl of 50µM riboflavin was added and the tubes were exposed to 200 W Philips fluorescent lamps for 10 min. The control tube contained an equal amount of buffer instead of a sample. The sample and its respective control were run together. At the end of the exposure, 1.0 ml of Greiss reagent was added to each tube and absorbance was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50 % of nitrite formation under assay condition [24].

Determination of catalase (CAT) activity

0.9 ml of 0.01M phosphate buffer, 0.1 ml of sample extract and 0.4 ml of 0.2M H_2O_2 was added. At 0 and after 60 sec, 2.0 ml of dichromate-acetic acid solution was added and tubes were kept in boiling water bath for 10 min, and the colour developed was read at 620 nm. Standards in the range of 1.2–6.0 µM were taken and processed as test and blank containing reagent alone. The activity of catalase was expressed as µmoles of H_2O_2 decomposed/min/mg protein or ml of serum [25].

Determination of glutathione peroxidase (GPx) activity

To 0.4 ml of 0.4M phosphate buffer, 0.2 ml of 4 mmol EDTA, 0.1 ml of 10 mmol NaN_3, 0.2 ml of 4 mmol reduced glutathione and 0.1 ml of 2.5 mmol H_2O_2 was added to two test tubes labeled as a test (T) and control (C). To the test added, 0.2 ml of sample and to the control added 0.2 ml of water. The contents were mixed well and incubated at 37 ^{O}C for 10 min. The reaction was arrested by adding 0.5 ml of 10 % TCA and the contents were centrifuged. To 1.0 ml of the supernatant, 3.0 ml of buffer and 0.5 ml of Ellman's reagent was added and the colour developed was read at 412 nm [25].

Determination of total reduced glutathione (GSH)

Standard glutathione solution of 0.2 to 1.0 ml (40–200 µg/ml) was used. Volume in all tubes was made up to 1.0 ml with distilled water. 0.5 ml of sample was precipitated with 2.0 ml of 5 % TCA. 1.0 ml of supernatant was taken after centrifugation. 0.5 ml of Ellman's reagent and 3.0 ml of 0.2M phosphate buffer was added to all the tubes. The absorbance was read at 412 nm within 2 min against the reagent blank. The amount of glutathione was expressed as µg/g tissue or ml of serum [26].

Statistical analysis

The data are expressed as a mean±SD Statistical comparison was done at significance level, $p<0.05$ using SPSS package version 10.0. One way ANOVA followed by post hoc analysis of LSD was performed.

RESULTS AND DISCUSSION

Disease index

Rats challenged with DMBA administration revealed severe loss in body weight, the appearance of chronic diarrhoea and fecal containing blood. The body weight of the rats with DMBA induced colitis began to decrease at 4th week after the initiation of DMBA. All the rats of both 10 mg and 20 mg group showed a steady decrease in body weight from the date of induction which was screened on 4th, 8th, 12th, 24th, and 32nd week. On 32nd week the weight loss was high in percentage (>25) when compared with control was listed in table 1. Disease index of both 10 mg and 20 mg was pooled together.

Table 1: Screening of disease activity index

DAI score	Weight loss (%)	Stool consistency	Occult/gross bleeding
0 w	None	Normal	Normal
4th week	1-7	Loose stools	Positive
8th week	7-15	Diarrhoea	Bleeding
12th week	15-20	Diarrhoea	Bleeding
24th week	20-25	Loose stools	Positive
32nd week	>25	Diarrhoea	Bleeding

The percentage change indicates the loss of weight as compared to control group. A non-parametric way of analysis was used to disease activity index.

Macroscopic scoring

The DMBA produce a severe inflammation of the distal colon and rectum as monitored by macroscopic damage. The inflammation was significantly heavier by week 4; and by the end of 32 w, inflammation in rats was double that of the controls. The weights of the colon and rectum in DMBA induced rats were 2 to 5 fold higher than control tissues. Macroscopic scoring of the colon and rectum showed edematous inflammation.

The clinical score was found to be 11.122±0.11 (colon), 10.524±0.016 (rectum) on 32nd week in 20 mg and 10.153±0.11 (colon), 8.612±0.016 (rectum) on 32nd week in 10 mg DMBA induced rats. The changes observed were clearly showed in table 2.

Table 2: Effect of DMBA on colon weight and macroscopic score in rats

Groups (n=3)	Colon weight	Macroscopic score	Rectum weight	Macroscopic score
Group I (control)	0.058±0.002	-	0.44±0.001	-
A) Group II (10 mg)				
4th week	0.080±0.003a*	1.231±0.034a*	0.053±0.002a*	2.110±0.021a*
8th week	0.098±0.004a*	3.352±0.019a*	0.062±0.003a*	4.127±0.019a*
12th week	0.145±0.004a*	5.556±0.011a*	0.082±0.002a*	5.960±0.015a*
24th week	0.187±0.005a*	8.996±0.013a*	0.110±0.001a*	7.173±0.014a*
32nd week	0.224±0.003a*c*	10.153±0.011a*c*	0.131±0.003a*c*	8.612±0.016a*c*
B) Group II (20 mg)				
4th week	0.092±0.003a*b*	3.323±0.021a*b*	0.061±0.004a*b*	2.159±0.019a*b*
8th week	0.136±0.002a*b*	4.876±0.010a*b*	0.086±0.003a*b*	3.741±0.015a*c*
12th week	0.192±0.003a*b*	6.598±0.013a*b*	0.118±0.001a*b*	7.157±0.016a*c*
24th week	0.245±0.003a*b*	9.234±0.012a*b*	0.145±0.003a*b*	9.813±0.015a*c*
32nd week	0.266±0.004a*b*d*	11.122±0.11a*b*d*	0.156±0.002a*b*d*	10.524±0.016a*b*d*

Values expressed as mean±SD (n 3)*-Significant at 5% (p<0.05), Group comparison: a) GII vs GI b) GIIB vs GIIA c) GIIA5 vs GIIA1, GIIA2, GIIA3 and GIIA4 d) GIIB5vs GIIB1, GIIB2, GIIB3and GIIB4

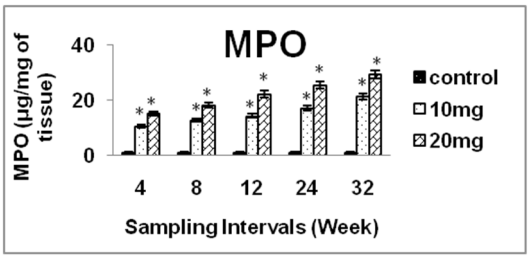

Fig. 1: MPO activity of colonic mucosa in experimentally induced colitis modelMyeloperoxidase activity: MPO–myeloperoxidase. *Values expressed as mean±SD (n 3) *-Significant at 5% (p<0.05)*

The effect of administration with DMBA 10 and 20 mg/kg on colon and rectum myeloperoxidase (MPO) activity is shown in fig. 1 and 2. The MPO activity is a marker for inflammation, significantly increased in ulcerative colitis model group. Both colon and rectum demonstrate the increased mucosal MPO concentration in rats. 20 mg and 10 mg group showed increased concentration on 32nd week when compared with control (Colon rectum= 29.34±0.31 and 21.41 vs 1.21µg/mg of tissue). Gradual increase was observed from 4th week on 32nd week the inflammatory effect was increased to 4 folds in both colon and rectum.

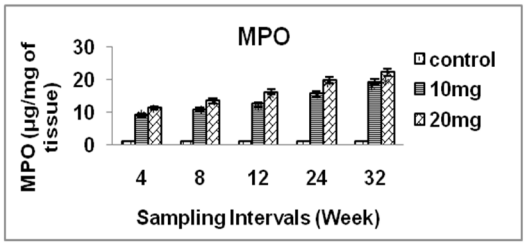

Fig. 2: MPO activity of rectum mucosa in experimentally induced colitis model, MPO–myeloperoxidase. *Values expressed as mean±SD (n 3) *-Significant at 5% (p<0.05)*

Assay of antioxidants in tissue samples

Table 3: Enzymatic antioxidant levels of colon and rectum tissues in DMBA induced rats

Groups	SOD		CATALASE		GPx	
	Colon	Rectum	Colon	Rectum	Colon	Rectum
Group I (Control)	6.59±0.05	5.11±0.04	21.53±0.23	19.48±0.15	7.21±0.02	10.22±0.02
Group II (induced)						
A) (10 mg)						
1) 4th	6.02±0.02a*	4.78±0.01a*	19.75±0.18a*	18.09±0.17a*	6.22±0.03a*	10.02±0.01a*
2) 8th	5.62±0.04a*	4.03±0.03a*	18.13±0.21a*	17.29±0.13a*	5.21±0.02a*	9.43±0.03a*
3) 16th	4.29±0.03a*	3.59±0.02a*	16.57±0.16a*	15.76±0.15a*	3.54±0.03a*	8.54±0.01a*
4) 24th	3.87±0.02a*	2.96±0.02a*	14.62±0.18a*	13.26±0.14a*	2.74±0.01a*	6.26±0.03a*
5) 32nd	3.10±0.01a*c*	1.66±0.03a*c*	13.32±0.19a*c*	11.21±0.15a*c*	1.29±0.02a*c*	5.29±0.02a*c*
B) (20 mg)						
1) 4th	5.36±0.02a*b*	4.22±0.03a*b*	19.12±0.17a*b*	17.51±0.14a*b*	5.69±0.03a*b*	9.74±0.03a*b*
2) 8th	3.93±0.01 a*b*	3.56±0.04 a*b*	17.79±0.15a*b*	16.51±0.19a*b*	4.32±0.04 a*b*	7.24±0.02 a*b*
3) 16th	2.63±0.03a*b*	2.13±0.02a*b*	15.24±0.19a*b*	14.36±0.21a*b*	2.94±0.02a*b*	6.32±0.01a*b*
4) 24th	1.54±0.02a*b*	1.79±0.01a*b*	13.15±0.18a*b*	11.23±0.19a*b*	2.14±0.02a*b*	5.13±0.02a*b*
5) 32nd	0.93±0.01a*b*d*	0.76±0.03a*b*d*	10.51±0.21a*b*d*	8.22±0.22a*b*d*	0.73±0.02a*b*d*	4.31±0.02a*b*d*

Values expressed as mean±SD (n 3)*-Significant at 5% (p<0.05), Group comparison: a) GII vs GI b) GIIB vs GIIA c) GIIA5 vs GIIA1, GIIA2, GIIA3 and GIIA4 d) GIIB5vs GIIB1, GIIB2, GIIB3and GIIB4. SOD-superoxide dismutase (SOD), CAT–catalase, GPx-glutathione peroxidise

In the development of intestinal damage, oxidative stress is involved in the aberrant immune and inflammatory responses. Though the precise mechanisms remain unclear, oxidative damage in the colorectal mucosa is considered an important process. In the present study, DMBA resulted in severe oxidative stress in the colorectal tissues of rats, as evidenced by reducing SOD content, CAT activity, GPx and GSH levels. Table 3 shows the enzymatic SOD, CAT and GPx levels were greatly reduced on 32nd week on both 10 mg and 20 mg group when compared with control. The periodical analysis was done on 4th, 8th, 12th, 24th and 32nd week from the date of induction. SOD, CAT and GPx showed a steady reduction. Non-enzymatic GSH levels were depicted in fig. 3 and 4. Induction of colitis produced a significant decrease in colorectal GSH content compared to control group (Colon 510±19 nmol/g *vs.* 1123±22 nmol/g Rectum 451±22 nmol/g *vs.* 1430±25 nmol/g) on 32nd week.

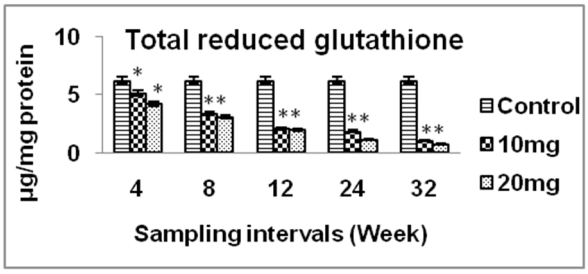

Fig. 3: GSH activity of colon mucosa in experimentally induced colitis model. GSH-total reduced glutathione. *Values expressed as mean±SD (n 3). *-Significant at 5% (p<0.05)*

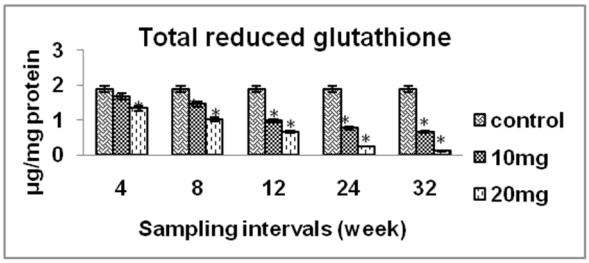

Fig. 4: GSH activity of rectum mucosa in experimentally induced colitis model, GSH-total reduced glutathione. *Values expressed as mean±SD (n 3) *-Significant at 5% (p<0.05)*

DISCUSSION

Inflammatory bowel disease is a disorder in which both autoimmune and immune-mediated disorders are involved. In the two forms especially in UC, an autoantigen named human tropomysin isolated form 5 (hTM5) plays an important role in the activation of humoral and cellular-mediated responses [28]. Modification of factors associated with ulcerative colitis results in the provision of relief to the patients. Among these factors, reactive oxygen species (ROS) plays an important role in the progression of the disease [29]. The present study identified important changes in the antioxidant status and myeloperoxidase levels of rats with ulcerative colitis induced with DMBA. Compared with healthy control rats induced group rats showed abnormal changes in body weight and antioxidant levels. These changes resulted indicates the association between ulcerative colitis and oxidation level. 7, 12-dimethylbenz anthracene (DMBA), is an immune-suppressor as well as a potent organ-specific carcinogen. It has been used extensively as a model carcinogen in cancer research it is also called as Bay-region dihydro diol epoxide, produced during cellular metabolism [30]. DMBA causes free radical changes, and a high correlation has been found to occur between the dose of administered DMBA and the levels on a target organ epithelial cells. It produces toxic and highly diffusible reactive oxygen species, capable of producing deleterious effects [31]. No articles are supporting DMBA induced colitis.

Reactive oxygen species are able to produce chemical modifications and to damage proteins, lipids, carbohydrates and nucleotides in the tissues. Reactive free radicals may damage cells by initiation of peroxidation that causes a profound alteration in the structural integrity and functions of cell membranes. Free radical has been implicated in the pathogenesis of several pathological disorders [32]. Free radicals are known to occur as natural by-products under physiologic conditions. However, their overproduction has been implicated in the pathogenesis of gut inflammation and intestinal injury in inflammatory bowel disease [33].

Animal experimental systems are particularly useful for the study of human ulcerative colitis. Since rats closely mimic human diseases, they have been selected in comparison to other animal models. In the present investigation, periodical analysis of DMBA induced ulcerative colitis in rats was done. As a result, the body weight has reduced tremendously, the colon-rectum weight was increase and the microscopic score was also huge and the percentage of the score was statistically significant (*P<0.05*). However, there was a severe body weight loss observed at the experiments end (32nd week), versus the control rats. The wet weight of the inflamed colon tissue is considered a reliable and sensitive indicator of the severity and extent of the inflammatory response [22]. In the present study, DMBA induced colitis significantly increased the wet weight of colon and rectum, clinical activity, gross lesion score and percentage of affected area compared with colitis control. Disease activity was steadily increasing from 4th week showed severe damage on 32nd week. It indicates that at fixed intervals the changes was observed. Since the intestine is in a constant state of controlled inflammation, thus amplification of the inflammatory response activates infiltration of inflammatory cells that triggers pathological responses and symptoms of IBD [34].

Myeloperoxidase is an enzyme present in neutrophils and at a much lower concentration in monocytes and macrophages. The level of MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue. Therefore, a measurement of MPO activity has been considered a quantitative and sensitive assay for acute intestinal inflammation. In addition, increased MPO activity has been reported to be an index of neutrophil infiltration and inflammation [35]. Induced dosage 10 mg and 20 mg group exhibited a significant increase in the MPO levels when compared to control rats. MPO measurements were steadily increased from 4th week till 32nd week. It shows the inflammation level.

Enzymatic and non-enzymatic antioxidants forms defence mechanism respectively against the deleterious effects of oxidative stress induced cell damage [36]. In IBD, oxidative stress plays a role in disease initiation and progression [37]. ROS attack the cellular macromolecules, thus disrupting epithelial cell integrity and triggering a mucosal recovery, especially in the case of impaired endogenous defence system [38]. In this work, DMBA induced ROS formation, as indicated by depletion of SOD, CAT, GPx and GSH when compared with control. Compared to 10 mg dose 20 mg dose showed a greater reduction. These findings are supported by previous findings [39, 40].

CONCLUSION

From the above, and taking together that UC is an immune related disease. The involvement of oxidative stress in ulcerative colitis its subsequent development is clearly increasing from 4th week and it reaches maximum on 32nd week. UC is a chronically recurrent inflammatory bowel disease of unknown origin. Oxidative stress has been implicated in the pathogenesis of ulcerative colitis is clearly pictured through above findings. It clearly proves that oxidative stress is a potential etiological and or triggering factor for UC, because the effects of reactive oxygen molecules have been well established in the inflammation process study from 4th week till 32nd week. In conclusion, the immunosuppressant drug DMBA has been found to cause colitis in the current study and the condition particularly elevated at 32nd week. This drug-induced model of

colitis may be further studied for unravelling previously unknown features of the recalcitrant disease namely IBD.

CONFLICT OF INTERESTS

Declared none

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