

Effects of Antioxidant Therapy on Leukocyte Myeloperoxidase and Cu/Zn-Superoxide Dismutase and Plasma Malondialdehyde Levels in Experimental Colitis

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The aim of the present study was to evaluate the effects of N-acetylcysteine (NAC) and L-carnitine (LCAR) supplementations on polymorphonuclear leukocytes myeloperoxidase (MPO) and Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and plasma malondialdehyde (MDA) in acetic acid (AA)-induced ulcerative colitis model. The mean polymorphonuclear leukocyte MPO and Cu/Zn-SOD activity was significantly higher in the colitis group than in the control group. Both NAC and LCAR pretreatment markedly decreased MPO and Cu/Zn-SOD activity compared to colitis group. AA administration significantly increased the levels of plasma MDA in comparison with controls. However, NAC and LCAR administration to the AA-treated rats significantly reduced the MDA levels compared to colitis group. In conclusion NAC and LCAR could be beneficial agents in restoring the circulating proinflammatory mediators.

INTRODUCTION

Ulcerative colitis is a chronic recurrent inflammatory bowel disease (IBD) of unknown origin. Oxidative stress is believed to be a key factor in the pathogenesis and perpetuation of the mucosal damage in IBD. In addition, neutrophils and monocytes in patients with active IBD have been shown to produce higher concentrations of oxygen-derived free radicals than those in controls. Excessive production of reactive oxygen species (ROS) could also be demonstrated for circulating phagocytic cells in patients with IBD [1] and was shown to be involved in several experimental models [2, 3]. However, the gut is potentially more exposed to oxidant injury due to the low concentration of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase), which are mainly localized in epithelial cells [4].

Two cytoplasmic enzymes, superoxide dismutase (SOD) and myeloperoxidase (MPO), protect the cell contents against oxidizing activity by destroying superoxide

anions ($^{\cdot}O_2$) and hydrogen peroxide (H_2O_2), respectively [5]. Also MPO is one of the indicators of inflammation and it is well correlated with neutrophil infiltration in various colitis models. SOD reduces the oxidative stress and the activation of mediators of inflammatory response [6].

Severe oxidative stress produces ROS and induces uncontrolled lipid peroxidation. The oxidation of polyunsaturated fatty acids is accompanied by the formation of a complex mixture of products including aldehydes, such as alkanals, alk-2-enals, alka-2,4-dienals, and malondialdehyde (MDA) [7, 8]. MDA is frequently used in the measurement of lipid peroxide levels. Elevated levels of MDA are shown in IBD also.

Some agents have already proved effective in experimental conditions, and clinically the antioxidants as superoxide dismutase and allopurinol were found to be of benefit in IBD. The efficacy of current standard therapy, for example, aminosalicylates, was found to be related to their antioxidant and scavenging action [9]. Compounds with antioxidant activity should therefore be investigated as potential therapeutic agents in IBD. We chose N-acetylcysteine (NAC) and L-carnitine (LCAR) as antioxidant agents.

The aim of the present study was to evaluate the effects of NAC and LCAR supplementations on polymorphonuclear (PMN) leukocytes MPO and Cu/Zn-SOD and

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plasma MDA in acetic acid (AA)-induced ulcerative colitis.

MATERIALS AND METHODS

Fourty male Wistar-albino rats weighing 210–240 g were used. The animals were housed under 12-hour light/dark cycles at a constant temperature (21–22°C) and fed on a standard rat chow and water *ad libitum*. The rats were randomly divided into four groups: Group I, control group ($n = 10$); Group II, colitis group ($n = 10$); Group III, colitis pretreated with NAC ($n = 10$); and Group IV, colitis pretreated with LCAR ($n = 10$).

Induction of colitis and treatment protocols

Colitis was induced by intracolonic instillation of 1 mL of 4% AA. After ether anesthesia a soft 6F pediatric catheter was inserted into the lumen of the colon *via* the anus for 6 cm and AA was carefully administered. Control rats were treated identically but instead of 4% AA, they received 1 mL 0.9% saline infusion.

NAC and LCAR were performed intraperitoneally one hour before the induction of colitis in Groups III and IV, respectively. Both agents were applied as 500 mg/kg in one milliliter. Blood samples were obtained for biochemical tests prior to sacrifice of the rats at 24 hour following the treatment. Under general anesthesia, all rats were sacrificed by cervical dislocation and then laparotomy was performed. The distal 8 cm of the colon was excised, freed of adherent adipose tissue, and opened longitudinally. The colon was immediately examined visually and observed for the damage.

Biochemical analysis

Isolation of polymorphonuclear (PMN) leukocytes

Blood was drawn from heart of rat with a heparinized disposable syringe, and mixed with 6% dextran in saline in a ratio of 4 : 1 and allowed to stand for 60 minutes at room temperature. The supernatant was removed and centrifuged at 275 g for 10 minutes. The resulting cell pellet was resuspended in 8 mL of 0.1 M phosphate buffer (pH 7.4), layered over 3 mL of Ficoll-Hypaque and spun at 450 g for 30 minutes at 4°C. The cell pellet that contained the PMN was then resuspended in phosphate buffer and 3 mL of erythrocyte lysing solution (0.87% NH_4Cl) was added. The cell suspension was centrifuged at 275 g for 5 minutes, the supernatant was discarded, the pellet was washed three times with Hank's balanced solution (HBSS) and resuspended in 1 mL of HBSS. Cell numbers in the final suspension were counted and the suspension was stored at -20°C .

Preparation of PMN homogenates

The PMN suspension was frozen at -20°C and thawed six times, then homogenized using a motor driven Teflon-glass homogenizer (9000 rpm for 5 min at 0°C).

The protein content of the homogenate was measured using Lowry's method [10].

Measurement of MPO activity

MPO activity was determined by a modification of the O-dianisidine method [11]. The assay mixture, in a cuvette of 1 cm path length, contained 0.3 mL 0.1 M phosphate buffer (pH 6.0), 0.3 mL 0.01 M H_2O_2 , 0.5 mL 0.02 M O-dianisidine (freshly prepared) in deionized water and 10 μL PMN homogenate in a final volume of 3 mL. The PMN homogenate was added at last and the change in absorbance at 460 nm was followed for 10 min. All measurements were carried out in duplicate. One unit of MPO is defined as that giving an increase in absorbance of 0.001 per min and specific activity is given as IU/mg protein.

Measurement of Cu/Zn-SOD activity

Cu/Zn-SOD activity was measured according to the method described by Fridovich [12]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye that was measured at 505 nm. Assay medium consisted of the 0.01 M phosphate buffer, 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer solution (50 mM CAPS, 0.94 mM EDTA, saturated NaOH) with pH 10.2, PMN homogenate, solution of substrate (0.05 mM xanthine, 0.025 mM INT) and 80 IU/L xanthine oxidase. Cu/Zn-SOD activity was expressed as IU/mg protein.

Measurement of MDA levels

Lipid peroxidation level in the plasma samples was expressed in MDA. It was measured according to procedure of Ohkawa et al [13]. The reaction mixture contained 0.1 mL sample, 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid, and 1.5 mL of 0.8% aqueous solution of TBA. The mixture pH was adjusted to 3.5 and volume was finally made up to 4.0 mL with distilled water and 5.0 mL of the mixture of *n*-butanol and pyridine (15 : 1,v/v) were added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm. Plasma MDA level was expressed as nmol/mL.

Measurement of protein levels

The protein concentration of the urine samples was measured by Spectronic-UV 120 spectrophotometer by the method of Lowry [10].

Statistical analysis

Statistical analysis was performed by using SPSS 9.0 version for Windows Operating System. The results were analyzed using one-way ANOVA test. The significance level was set at $P < .05$.

TABLE 1. The effects of NAC and LCAR on PMN leukocyte MPO and Cu/Zn-SOD activity. Note: data are presented as mean \pm SD; PMN, polymorphonuclear; SOD, superoxide dismutase; MPO, myeloperoxidase; NAC, N-acetylcysteine; and LCAR, L-carnitine.

	PMN leukocyte MPO activity (IU/mg protein)	PMN leukocyte Cu/Zn-SOD activity (IU/mg protein)
Control group ($n = 10$)	$0.20 \pm 0.02^*$	$0.28 \pm 0.02^*$
Colitis group ($n = 10$)	$0.68 \pm 0.02^{**}$	$0.44 \pm 0.04^{**}$
NAC pretreatment group ($n = 10$)	0.41 ± 0.08	0.18 ± 0.02
LCAR pretreatment group ($n = 10$)	0.38 ± 0.06	0.33 ± 0.03

* $P < .05$, significantly different from treatment groups.

** $P < .001$, significantly different from control and treatment groups.

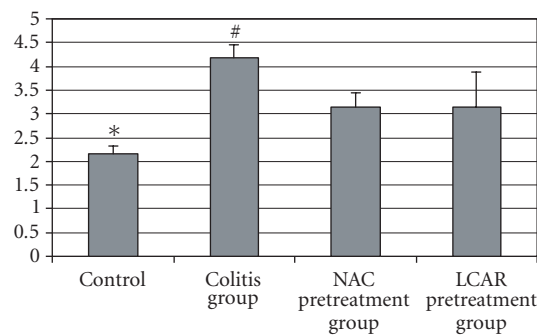


FIGURE 1. Plasma MDA (malondialdehyde) levels in AA-induced colitis in rats. Data are mean \pm SD of 10 rats per group. #, colitis group was significantly different from other groups ($P < .001$); *, control group was significantly different from pretreatment groups ($P < .001$).

RESULTS

The rats treated with acetic acid developed acute colitis at the incidence of 100%. During the study none of the rats died or were excluded from the study for any reason. The mean PMN leukocyte MPO and SOD activities were significantly higher in the colitis group than in the control group. Both NAC and LCAR pretreatment significantly decreased MPO and SOD activities compared to colitis group ($P < .001$) (Table 1). Furthermore, when control and treatment groups were compared by means of MPO and SOD activities, MPO activities were found significantly higher in treatment groups, while SOD activity was found significantly lower in LCAR group compared to the control. SOD activity in the NAC treatment group was also found markedly higher than the control group (Table 1). AA administration significantly increased the levels of plasma MDA in comparison with controls. However, NAC and LCAR administration to the AA-treated rats significantly reduced the MDA levels compared to colitis group ($P < .001$). When treatment groups and the control were compared, MDA levels were found significantly higher in NAC and LCAR groups than in the control group (Figure 1).

DISCUSSION

Many factors have been implicated in the pathogenesis of UC, such as neutrophil infiltration and overproduction of proinflammatory mediators including cytokines, arachidonate metabolites, and ROS [14, 15]. The tissue injury produced by neutrophils and macrophages has been attributed to their ability to release ROS, nitrogen metabolites, cytotoxic proteins, lytic enzymes, and cytokines as well as their disrupting effects on epithelial integrity [16, 17]. Since abnormal oxidative metabolism is of central importance in active UC, increased attention has been focused on the role of free radicals in UC [18, 19]. Increased production of ROS from stimulated inflammatory cells as well as lipid peroxidation by-products has been demonstrated in colorectal biopsy specimens of patients with UC [18, 19, 20].

The protective effects of various antioxidant agents such as vitamin E and selenium [21], Zolimid and AEOL11201 [22], sodium ferulate [23], and trimetazidine [24, 25] against the oxidative injury in experimental colitis models were investigated. Additionally, ameliorating effects of topical use of propionyl-LCAR on the histopathology of ulcerative colitis patients were demonstrated in two

different studies [26, 27]. We also showed the beneficial effects of NAC against the histopathologic and oxidant injury in experimental colitis induced by acetic acid in a recent article [28].

MPO, an enzyme found predominantly in activated PMN leukocytes, is commonly used to quantify acute colonic inflammation [29]. On the other hand, the correlation between MPO activities and the histological analysis of neutrophil infiltration of the colon was reported [30]. Colonic tissue MPO activities were shown to increase and following the application of various anti-inflammatory and antioxidant agents, tissue MPO levels were demonstrated to decrease in the recent experimental colitis studies [31, 32, 33, 34]. However, there is not enough data about the level of MPO-derived circulating PMN leukocytes in ulcerative colitis. In our study, the mean MPO activity was significantly higher in the colitis group than in the control group. NAC and LCAR administration to the AA-treated rats significantly reduced the MPO activity compared to colitis group.

Lipid peroxidation augments tissue damage in ulcerative colitis models. Girgin et al showed that MDA levels of the colon increased in the chronic colitis rat model [24]. Various antioxidant and anti-inflammatory agents used in the treatment of the ulcerative colitis were shown to decrease MDA levels [35, 36, 37, 38]. On the other hand, while it has been shown that colonic MDA levels are increased both in human and experimental studies, plasma MDA changes are not understood enough. However, we determined in this study that plasma MDA levels were increased in colitis group and NAC and LCAR decreased the levels of MDA.

SOD is an important protective system that accelerates the dismutation of superoxide anion radicals to hydrogen peroxide and acts as a primary defense, as it prevents further generation of free radicals. Results of the studies examining the status of the antioxidant enzyme SOD in experimental colitis are controversial. Kuralay et al showed that tissue SOD levels were elevated in response to oxidative stress in experimental colitis model, they were decreased by antioxidant agents [25]. However, angelica sinensis and sodium ferulate have been demonstrated to restore reduced colonic SOD levels in experimental colitis [23, 33]. Kruidenier and colleagues demonstrated that colonic mucosa Cu/Zn-SOD and Mn-SOD levels were higher than the control levels in patients with inflammatory bowel disease [39]. On the other hand, Verspaget et al demonstrated lower activity of SOD in peripheral blood neutrophils in ulcerative colitis patients [40]. Our study found that neutrophil-related Cu/Zn-SOD levels increased in colitis group and decreased after antioxidant therapy. This increase in colitis group may be explained by the response against oxidative stress.

Our findings indicate that the NAC and LCAR supplementation to the rats with AA-induced colitis can significantly attenuate the increase in PMN leukocytes Cu/Zn-SOD and MPO levels and plasma MDA levels; thus NAC

and LCAR protect the organism from AA-induced lipid peroxidation. In conclusion NAC and LCAR could be beneficial agents in restoring circulating proinflammatory mediators.

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