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Effect of N-acetylcysteine on Nonsteroidal anti-inflammatory drugs induced colitis in rats

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Abstract
The aim of this study is to evaluate the effect of NAC on NSAIDs induced colitis in rats.
Model of colitis was induced by subcutaneous Indomethacin prepared in 5% sodium bicarbonate administrated at a dose rate of 9 mg/kg for two days at 24 h intervals. NAC (500mg/Kg body weight po) was administrated for seven consecutive days beginning 24 h after the first Indomethacin injection. Colon injury was assessed by body weight loss, colon weight / length ratio, macroscopic damage, histological study, as well as by biochemical measurement of reduced glutathione (GSH), lipid Peroxides and superoxide dismutase (SOD) activity in the colon tissues. The results of this study showed that NAC administration didn't cause decrease in body weight loss, colon weight/length ratio, macroscopic and microscopic colon damage scores caused by administration of Indomethacin. The levels of GSH were decreased. SOD activity was increased and lipid peroxides levels were drcreased in the colon tissue of NAC treated group comparing with Indomethacin control group, but no statistical significance was observed (p>0.05). In this study conclude that NAC didn't ameliorate colitis induced by Indomethacin in this dose regimen.

Keywords: colitis, indomethacin, n-acetylcysteine, macroscopic score, histological study, superoxide dismutase, reduced glutathione, lipid peroxides

Introduction
Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) are well known for causing inflammation and ulcerative disease of the upper gastrointestinal tract [1]. Recently, it has become evident that the small and large intestines are also adversely affected by NSAID ingestion [2]. The effects of NSAIDs on the large intestine include inflammation, bleeding, ulceration, and perforation [3].The mechanism whereby NSAIDs could lead to colonic inflammation is unclear. Whether it is due to local or systemic mechanism is still controversial [4]. There are several mechanisms by which NSAIDs can cause gastrointestinal damage: systemic by inhibition of prostaglandin synthesis and uncoupling mitochondrial phosphorylation, but also topical, because NSAIDs are weak acids that can disrupt the epithelial cell barrier. Additional potential harmful mechanisms include vasoconstriction and the activation of platelets [5]. N-Acetylcysteine (NAC) is an exogenous antioxidant, which works as a free radical scavenger. It is also a glutathione precursor regarded as one of the most important intracellular antioxidants [6]. Moreover, NAC has been purported to have anti-inflammatory properties [7]. It has been used for more than 50 years, there are still many controversies surrounding it as a medicine as well as a dietary supplement [8]. NAC is being studied and utilized in conditions characterized by decreased GSH or oxidative stress such as HIV infection, cancer, and heart disease. Because of its hepato-protective activity, intravenous and oral administrations of NAC have been used extensively in the management of acetaminophen poisoning [9].
The aim of this study is to evaluate the effect of NAC on colitis induced by NSAIDs in rats.

Materials and Methods
Animals and experimental design
Female and male wistar albino rats weighing 160-290 g were purchased from the Scientific Research Center, Damascus, Syria. The animals were provided with ad libitum feed and water. The animals were kept at controlled environmental conditions (temperature 23 ± 2 °C, humidity 55 ± 15%, lighting regimen of 12h light:12-h dark). They were acclimatized for one week before any experimental. All methods in this study were performed in concordance with regulatory guidelines on the care and use of laboratory animals; National Research council [NRS] 2011. Guide for the Care and Use of Laboratory Animals. 8th Washington: National Academies Press. Animals were randomly divided into four groups:

Group I: normal control group (6 rats in this group) received oral vehicle (physiological saline)

Group II: Indomethacin control group (7 rats in this group) received subcutaneous Indomethacin prepared in 5% sodium bicarbonate, administered at a dose rate of 9 mg/kg for two days at 24h intervals. It also received oral vehicle (physiological saline).

Group III: NAC treated group (8 rats in this group) received NAC dissolved in physiological saline (500 mg/kg body weight po) for seven consecutive days beginning 24 h after the first Indomethacin injection. This group was given subcutaneous Indomethacin prepared in 5% sodium bicarbonate and administered at a dose rate of 9 mg/kg for two days at 24h intervals.

On day eight, each sub group of animals across all groups was sacrificed.
The colon was removed and was opened longitudinally, tissues were washed in saline solution, and any macroscopic change was checked. A precise evaluation of the lesions was made after each specimen was fixed in 10% formalin.
Colon tissue was collected and stored at -80 °C till further analysis.

Clinical findings
During the study, rats were checked daily for body weight, behavioral changes, food intake, intestinal bleeding and stool consistency.
The body weight of animals was measured at regular time intervals from day 0 to 7 and change of body weight (%) was calculated.

Colon weight/length ratio
The length and weight of the colon was measured for the estimation of:
Weight of colon (g)/length of the colon (cm) ratio

Macroscopic characters [10].

Table 1: Macroscopic inflammation assessment of the colon

Score	Macroscopic score
0	No visible change
1	Hyperemia at sites
2	Lesions having diameter l mm or less
3	Lesions having diameter 2 mm or less (number < 5)
4	Lesions having diameter 2 mm or less (number 5–10)
5	Lesions having diameter 2 mm or less (number > 10)
6	Lesions having diameter more than 2 mm (number < 5)
7	Lesions having diameter more than 2 mm (number 5–10)
8	Lesions having diameter more than 2 mm (number >10)

Histopathological observations
A portion of the colon specimen from each rat was fixed with 10% formalin, embedded in paraffin wax and cut into sections of 5mm thickness. The sections were stained with hematoxylin and eosin (H and E) dye for histopathological observations. The following histological features were examined by an unbiased pathologist (AM) blinded to the experimental design: grade and type of inflammation, extension of inflammation throughout the gastrointestinal wall (mucosa, submucosa, muscular layer and serous membrane), presence of Lymphocytic Follicle/aggregate, Necrosis, Granuloma, Cryptitis, Crypt abscess and epithelial lesions (erosions, ulcers) [11].

Biochemical estimations
Accurately weighed tissues from the colon were homogenized in cold phosphate buffered saline [pH 7.4, 50 mM] to prepare 10% homogenate and the suspension was divided into three portions. One part of tissue suspension was mixed with 0.2 ml 5% trichloroacetic acid for measurement of GSH levels, second part of tissue suspension was used for measurement SOD activity.
One and two parts of tissue.
Homogenate were centrifuged at 10000g for 20 min at 4º C and supernatant was used for assay GSH levels and SOD activity.
The remaining of third portion of tissue homogenate was used for the estimation of Lipid peroxides levels.

Assay of reduced glutathione (GSH)
Reduced glutathione (GSH) was measured by reaction with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) to give a compound that absorbs at 412nm (Ellman's method). In short, each sample cuvette contained 2ml 0.6mM DTNB in 0.2M sodium phosphate, pH 8.0, 0.1-0.2ml supernatant fraction, and 0.2M phosphate buffer to a final volume of 3ml. (Measurement of the pH in the cuvette showed that the buffer capacity was sufficient to neutralize the trichloroacetic acid present in the sample, and assay of known amounts of GSH in the presence of 0.1-0.2ml 5% trichloroacetic acid demonstrated that this substance did not interfere with the procedure in any other way.) The reference cuvette contained 0.1-0.2ml 5% trichloroacetic acid instead of sample, and the reaction was started by the addition of supernatant to the sample cuvette [12]. It is expressed as µM of GSH per gram of tissue.

Assay of superoxide dismutase (SOD) activity
The recommended procedure is as follows. First, a certain amount of pyrogallol solution (60 mM in 1 mM HCl, 37 °C) was thoroughly mixed with pH 7.4 Tris-HCl buffer (0.05 M, 37 °C) containing 1 mM Na2EDTA (to remove metal ions, which may catalyze the reaction); the total volume was adjusted to 3000 µL using the buffer. The A325 nm value of the mixture without a sample was measured every 30 s for 5 min at 37 °C. Second, an amount of pyrogallol solution equal to that used in the first step was added to a mixture with a sample, and the total volume was adjusted to 3000 µL using the buffer.
Enzyme activity which corresponds to amount of enzyme that inhibits auto-oxidation of pyrogallol by 50% was calculated and expressed per mg of protein [13].

Assay of lipid peroxidation (TBARS)
Lipid peroxidation, an indicator of mucosal injury induced by reactive oxygen species was measured as thiobarbituric acid reactive substance. Briefly, 0.5 ml of small intestinal tissue homogenates prepared were reacted with 2 ml of TBA reagent containing 0.375% TBA, 15% trichloroacetic acid and 0.25 N HCl. Samples were boiled for 15 min, cooled and centrifuged. Absorbance of the supernatants was measured by spectrophotometer measured at 532 nm [14, 15]. The concentration of MDA was calculated by the absorbance coefficient of MDA–TBA complex (1.56×10^5 M/cm) and expressed in µmol/100 g of tissue.

Statistical analysis
Data analyses were achieved using a software program Graph Pad Prism version 8. Data were expressed as mean ± SEM, and different groups were compared using one way analysis of variance (ANOVA) followed by Sidak test for multiple comparisons for parametric data, and Kruskal–Wallis test followed by Dunn test for multiple comparisons for non-parametric data and parametric data that have shown non normal distribution. P values less than 0.05 were considered statistically significant.

Results
Clinical findings, general observation and body weight change
After 24 h of administration first dose of Indomethacin, animals developed soft feces, weakness, decreased food intake and progressively body weight loss. All these

symptoms reached a maximum at three days from first dose of Indomethacin, and then these symptoms started to decrease gradually.

The group treated with NAC didn't reveal any progress of these symptoms, some rats of these groups revealed diarrhea and intestinal bleeding.

Compared with that of the normal control group which revealed increase in body weight (1.63%), the body weight of the Indomethacin control group at the end of experiment was reduced by (-4.58%) with statistical significance comparing with normal control group (p=0.0402).

NAC treated group (500 mg/kg) revealed decrease in body weight (-4.89%) with no statistical significance comparing with Indomethacin control group (p=0.9119).

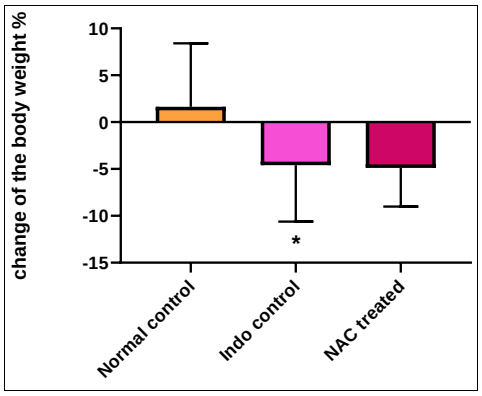

Fig 1: Effect of N-acetylcysteine on the body weight in Indomethacin induced colitis in rats.

Values are given as mean± S.E.M. values are statistically significant at *P<0.05 between Normal and Indo control groups

Abbreviations

Indo=Indomethacin,

NAC=N-acetylcysteine

Colon weight / length ratio

Colon weight / length ratio is indirect reliable marker of the inflammation. The increase in this ratio in Indomethacin control group was observed, there was statistical significance comparing with normal control group (p=0.0372). NAC treated group (500 mg/kg) didn't decrease colon weight / length ratio, the increase in this ratio was observed in this group. No statistical significance comparing with Indomethacin control group (p= 0.8687).

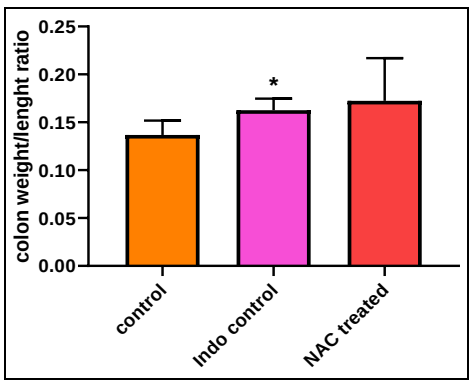

Fig 2: Effect of N-acetylcysteine on colon weight/length ratio in Indomethacin induced colitis in rats.

Values are given as mean± S.E.M. values are statistically significant at *P<0.05 between Normal and Indo control groups

Abbreviations

Indo=Indomethacin,

NAC=N-acetylcysteine

Macroscopic score

The most sections of the colon in normal control group revealed hyperemia at sites. Subcutaneous injection of Indomethacin produced damage in the colon. Erosion, edema, hemorrhagic spots were noticed. Some of these lesions have diameter greater than 2mm, thus the morphological score in the Indo control group was significantly increased (p=0. 0.0134) as compared to normal control group. Administration of NAC didn't reduce the severity of the gross lesion induced by indomethacin in the colon. No statistical significance comparing with Indomethacin control group (p=0.9962).

Table 3: Macroscopic score of different experimental groups

Group Macroscopic Score	Normal control	Indo control	NAC treated (500mg/kg)
0	1(16.67%)		
1	5(83.33%)	2(28.57%)	3(37.5%)
2		1(14.29%)	
3		1(14.29%)	1(12.5%)
4			
5			
6		3(42.86%)	4(50%)
7			
8			

Abbreviations: Indo=Indomethacin, NAC=N-acetylcysteine

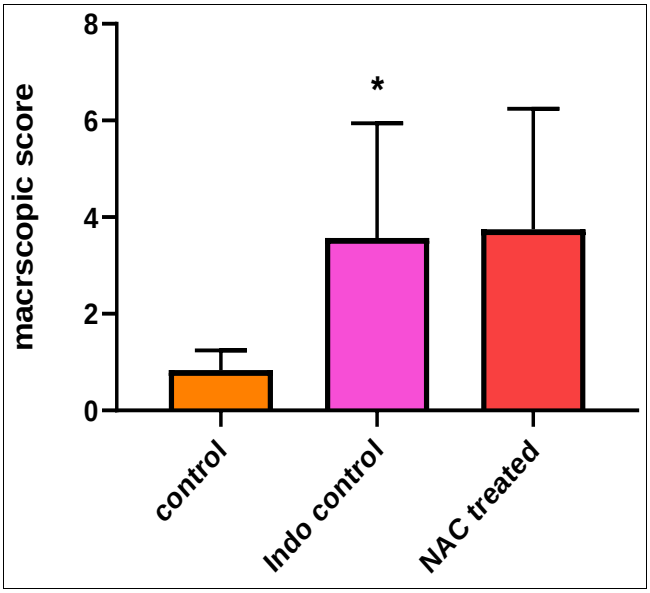

Fig 3: Effect of N-acetylcysteine on macroscopic score in Indomethacin induced colitis in rats.

Values are given as mean± S.E.M. values are statistically significant at *P<0.05 between Normal and Indo control groups

Abbreviations

Indo=Indomethacin,

NAC=N-acetylcysteine

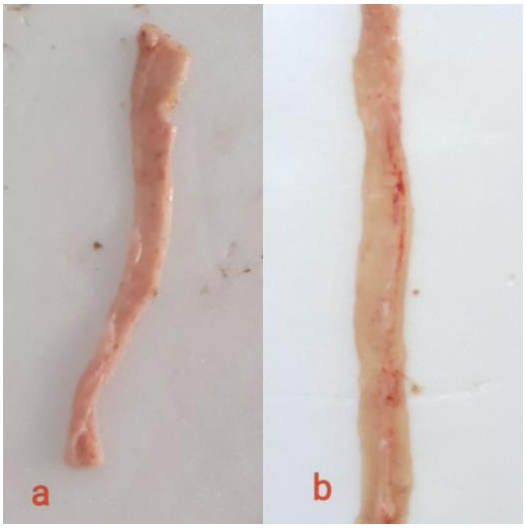

Fig 4: Macroscopic appearance of the colon in Normal control group. **a-** score 0**, b-**score 1

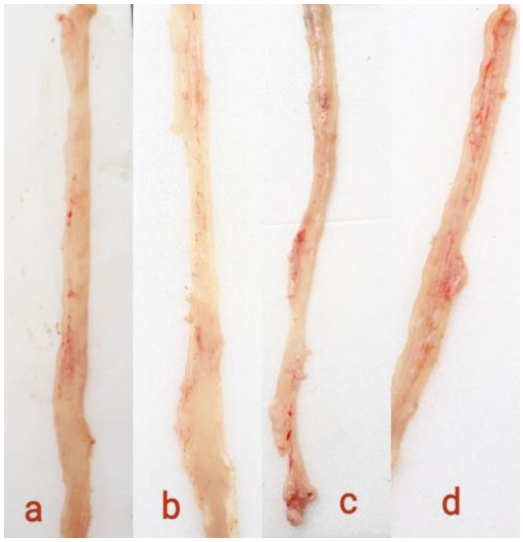

Fig 5: Macroscopic appearance of the colon in Indomethacin control group. **a-** score 1**, b-** score 2**, b-** score 3**, d-** score 4

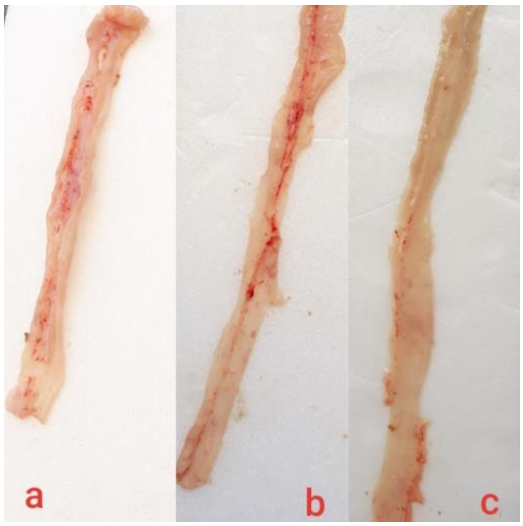

Fig 6: Macroscopic appearance of the colon in N-acetylcysteine treated group. **a-** score 1**, b-** score 3**, c-** score 6

Microscopic score

Some colonic tissue of normal animals showed increased inflammatory cells infiltration. In addition some of them showed transmural lymphocytic aggregate, or in submucosa layer. 50% of rats from this group, the colonic tissue showed an intact architecture.

On the other hand the colonic sections of Indomethacin control group revealed increased inflammatory cells infiltration, transmural inflammation, lymphocytic aggregate, cryptitis and erosion. There was statistical significance comparing with normal control group (p= 0.0237). NAC administration didn't significantly reduce the severity of inflammation and injury induced by Indomethacin. There was no statistical significance comparing with Indomethacin control group (p=0.5377).

Table 4: Microscopic score of different experimental groups.

Group Microscopic Score	Normal control	Indo control	NAC treated (500mg/kg)
0	3 (50%)		
1			1(12.5%)
2	2 (33.33%)	3(42.86%)	3(37.5%)
3	1 (16.67%)	2 (28.57%)	2(25%)
4			2(25%)
5		2(28.57%)	
6			
7			
8			
9			
10			
11			

Abbreviations: Indo=Indomethacin, NAC=N-acetylcysteine

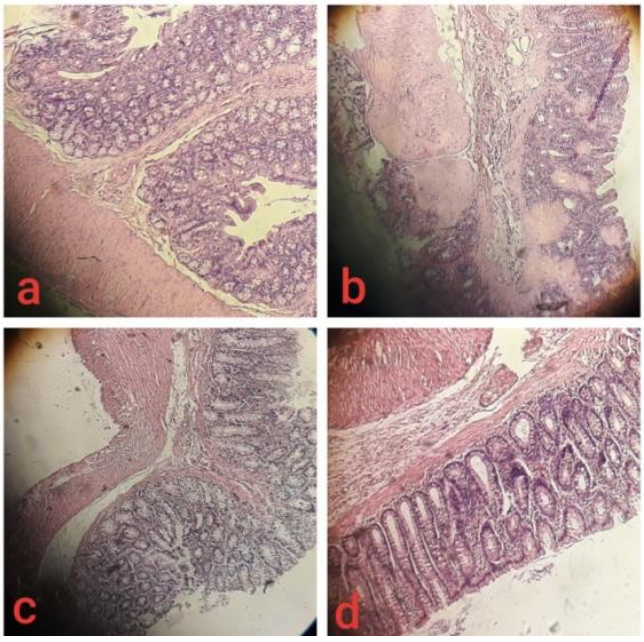

Fig 7: Histological appearance of colon tissue sections, original magnification ×10

A- Normal control group (grade 0) shows an intact architecture, B- Indomethacin control group (grade 5) shows cryptitis, transmural inflammation and lymphocytic aggregate.

C- N-acetylcysteine treated group (grade 2) shows an increased inflammatory cells infiltration, D- N-acetylcysteine treated group (grade 3) shows an increased inflammatory cells infiltration and lymphocytic aggregate

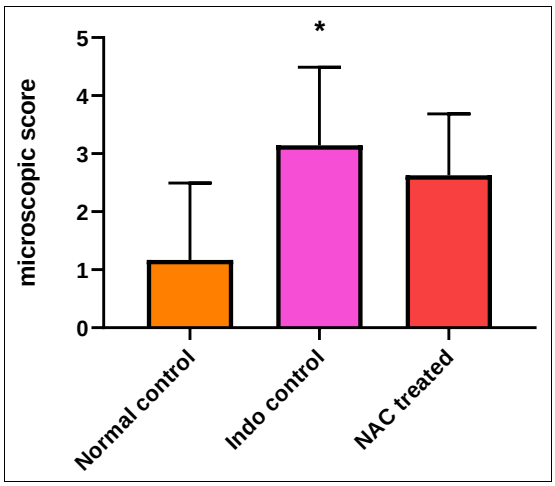

Fig 8: Effect of N-acetylcysteine on microscopic score in Indomethacin induced colitis in rats.

Values are given as mean± S.E.M. values are statistically significant at *P<0.05 between Normal and Indo control groups

Abbreviations
Indo=Indomethacin,
NAC=N-acetylcysteine

Biochemical assays
Indomethacin induced oxidative stress in the colon, which was evaluated by lipid peroxidation, SOD activity and GSH levels. Indomethacin increased the levels of lipid peroxides, decreased SOD activity and GSH levels in the colon, there was statistical significance comparing with normal control group (SOD: p= 0.0295, GSH: p= 0.0143, Lipid peroxides: p= 0.0052). NAC treated group (500 mg/kg) increased SOD activity, decreased the levels of lipid peroxides, but the levels of GSH was decreased. There was no statistical significance comparing with Indomethacin control group (SOD: p= 0.7712, GSH: p= 0.2118, Lipid peroxides: p= 0.3312).

Table 5: Effect of NAC on lipid peroxides, GSH and SOD activity in Indomethacin induced colitis in rats

Group Parameter	SOD activity	GSH levels (µM/g of tissue)	Lipid peroxides (µmol/100 g of tissue)
Normal control	0.154833±0.051699	2.623333±0.538576	0.46±0.1520
Indo control	0.021857±0.018313*	1.69285±0.139569*	0.6586±0.1145**
NAC treated	0.07875±0.07875	1.26625±0.218141	0.5213±0.1301

Values are given as mean± S.E.M. values are statistically significant at *P<0.05, **P<0.01 between normal and Indo control groups

Abbreviations
Indo=Indomethacin,
NAC=N-acetylcysteine,
GSH=Glutathione,
SOD=superoxide dismutase

Discussion
Indomethacin is a potent nonsteroid anti-inflammatory drug of proven effectiveness in man and in animals. Similar to other anti-inflammatory agents, indomethacin has been reported to produce gastrointestinal irritation and ulceration in man as well as in animals [16]. Indomethacin induces small intestinal and colonic ulceration in a dose-dependent fashion in rodents [17]. In this study Indomethacin-induced inflammation in the colon tissues, as evidenced by body weight loss, reduction in food intake, increases in colon weight / length ratio, changes in biochemical parameters which include depletion of GSH, increased lipid peroxides levels and decreased SOD activity. The macroscopic results revealed erosion, edema, and hemorrhagic spots. The microscopic score revealed increased inflammatory cells infiltration, transmural inflammation, lymphocytic aggregate, cryptitis and erosion. This study highlights the effect of N-acetylcysteine on Nonsteroidal Anti-Inflammatory Drugs induced colitis in rats. The effect of NAC on body weight improvement wasn't observed, in addition NAC administration didn't reduce the severity of the gross lesion in the colon which was evidenced by colon weight/length ratio, macroscopic and microscopic score. Regarding of the effect of NAC on antioxidant status, NAC in both doses decreased lipid peroxidation, increased SOD activity, but no statistical significance was observed when these findings were compared with Indo control group. Although NAC is a glutathione precursor, the effect of NAC on GSH levels in the colon wasn't observed, they were decreased in the colon tissues with no statistical significance comparing with Indomethacin control group. The decrease in the levels of GSH indicating that there was increase in the oxidative stress. This finding is agreement with SPRONG *et al* who proved that high doses of NAC aggravate LPS toxicity, the decrease in GSH at 6 h and 12 h after LPS injection, strongly suggest further oxidation of GSH by oxidative stress induced by high-dose NAC. This effect of NAC is supported by the considerable literature reporting that low molecular-weight thiols are pro-oxidants as well as antioxidants [18]. The previous findings weren't in harmony with several authors who have described the effect of NAC on colitis induced in animals. SIDDIQUI *et al* confirmed that treatment of Trinitrobenzenesulfonic acid (TNBS) induced colitis with NAC plus 5-aminosalicylic acid (5-ASA) was superior to treatment with either agent alone in reducing colonic inflammation and in promoting mucosal repair [19]. Cetinkaya *et al* proved that the NAC administration intraperitoneally or intrarectally to the rats with acetic acid induced colitis can reduce the extent of colonic mucosal injury, attenuate the increase in Myeloperoxidase (MPO) activity and Malondialdhyde (MDA) levels and restore diminished antioxidant enzymes and substance such as SOD, catalase (CAT) and GSH [20]. Wang *et al* showed that Dietary supplementation with NAC can alleviate acetic acid (AA) induced colitis in a porcine model through regulating anti-oxidative responses [21]. URAZ *et al* showed that NAC inhibited not only oxidant damage but also proinflammatory cytokines, and improved the colonic inflammation induced by AA in rats [22]. Amrouche-Mekkioui *et al* showed the role of NAC as a

scavenger of phagocytes-derived reactive oxygen species in dextran sodium sulfate (DDS) colitis, suggesting that NAC might be protective in oxidative inflammatory bowel disease and colorectal cancer [23]. Azooz *et al* showed that NAC has anti-inflammatory effects in the acute phase of TNBS-niduced colitis [24].

Conclusion

In this study conclude that NAC didn't ameliorate colitis induced by Indomethacin in this dose regimen, suggesting more investigations about the effect of NAC on this model of colitis using NAC in different doses.

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