ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Effect of Ethanol Consumption on Colon of Normal Rats as Well as Rats with Experimentally-Induced Ulcerative Colitis

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Manuscript Info

Manuscript History:

Received: 12 February 2015
Final Accepted: 22 March 2015
Published Online: April 2015

Key words:

Ethanol Inflammatory bowel isease Oxidative stress Ulcerative colitis

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Abstract

The concept that environmental factors may alter disease pattern has been an area of controversy in inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease. Ethanol consumption is associated with oxidative stress in multiple tissues in vivo. Ethanol and its metabolites are known to affect the gut barrier function, being a potential trigger for flare in IBD. The present study was conducted to evaluate the effect of moderate ethanol consumption on normal colon and on N-ethylmaleimide (NEM)-ulcerated colon in rats. To achieve this goal, rats were classified into four groups, namely normal control group receiving only vehicles, ethanol control group receiving only ethanol, UC control group receiving only NEM, and ethanol/UC group receiving both ethanol and NEM. At the end of the experiment, animals were sacrificed and colons isolated. A portion of each colon was allowed to harden in formalin solution in saline for histopathological study, and another portion was homogenized in normal saline and tissue homogenates were used for determination of reduced glutathione (GSH), total nitrate/nitrite (NO_X), myeloperoxidase (MPO) and tumor necrosis factor-α (TNF-α) contents. Ethanol consumption in normal rats significantly decreased colon length and GSH content, and significantly increased NO_X and TNF-α contents as compared to normal control rats. Additionally, ethanol consumption in rats with experimental UC significantly increased colon NO_X and TNF-α contents as compared to UC control rats. Results of histopathological study strongly supported results of biochemical estimations. Although ethanol consumed in moderate doses was reported to offer protection against some diseases, these data suggested that ethanol consumption even in moderate doses has harmful effects on normal colon and may worsen UC disease progression.

INTRODUCTION

The human colon is a dynamic organ, involved in a vast array of functions. The primary function of the colon is to absorb water and electrolytes from undigested foods and to store waste-products until excreted. The colon is viewed as the preferred absorption site for oral administration of protein and peptide drugs because of the relatively low proteolytic enzyme activities in it **(Yang et al., 2002).** Although it is not an organ essential to life, the colon still plays a major role in maintaining the overall health of the human body.

Inflammatory bowel diseases (IBDs) are recognized as important causes of gastrointestinal disease in children and adults **(Kirsner and Shorter, 1982).** They are chronic relapsing inflammatory disorders of the gastrointestinal tract **(Geier et al., 2007).** IBDs are conventionally divided into two major subtypes, namely

ulcerative colitis (UC) and Crohn's disease (CD), named according to their features. UC is a continuous superficial mucosal inflammation that is limited to the colon and rectum, while CD is a trans-mural inflammatory process that affects any part of the digestive tract from mouth to anus **(Carty and Rampton, 2003)**. IBDs are believed to result from the interaction of genetic, environmental and autoimmune factors **(Fiocchi, 1998).**

The important environmental social factor that can affect IBD is alcohol consumption. It has been associated with a wide variety of deleterious health effects such as liver disease and cardiomyopathy **(Klatsky, 2007).** In addition, acute and chronic alcohol consumption has been shown to modify the immune system and could therefore play an important role in IBD. Acutely, alcohol consumption has been shown to inhibit the immune system by decreasing T cell activity and interleukin IL-12 levels in healthy controls **(Mandrekar et al., 2004).** Alcohol can also increase monocyte production of anti-inflammatory cytokines such as IL-10 **(Norkina et al., 2007).** Chronically, alcohol increases liver Kupffer cell activity and is associated with increased generation of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), IL-1 and IL-6 **(Khoruts et al., 1991).** Alcohol has also been shown to acutely disrupt gut barrier function, and can increase intestinal permeability in human subjects **(Keshavarzian et al., 1994)** to which patients with IBD are particularly susceptible **(Wyatt et al., 1997).** Although alcohol has a variety of effects on the immune system, alcohol consumed in moderation may offer protection against some diseases and alcohol consumption in IBD, especially in moderate consumption, has rarely been examined in previous studies that prompted us to further explore the potential effects of ethanol on colon rats and on NEM- induced ulcerative colitis rats.

Several mediators have been implicated in the pathogenesis of IBD, including eicosanoids, cytokines, nitric oxide, reactive oxygen species and platelet activating factor **(Sartor, 1997)**. There seems to be a disturbed balance between pro-inflammatory and anti-inflammatory cytokines. Increased levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 were detected in the colonic mucosa of patients with IBD **(Matsuura et al., 1993; Andus et al., 1995; Niessner and Volk, 1995)**. Reactive oxygen species (ROS) include superoxide radical anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) as well as oxidants derived via myeloperoxidase (MPO), such as hypochlorous acid (HOCl) and N-chloramines (RNHCl). Myeloperoxidase (MPO) is an enzyme restricted mainly to polymorphnuclear neutrophils (PMNs) and hence its activity reflects the amount of PMN infiltration into the organ **(Xu et al., 2008).** It plays an important role in the production of oxidants by neutrophils, which are a potential source of ROS **(Dib et al., 2002).** Reduced glutathione (GSH) represents the main non-enzymatic free radical scavenger exerting antioxidant effect in the gastrointestinal mucosa either directly or acting as substrate for its related detoxifying enzymes **(Loguercio and Di Pierro, 1999)**. The depletion of GSH, as a consequence of enhanced production of free radicals, is considered an important criterion of colonic damage **(Loguercio et al., 1996; Camuesco et al., 2006; Arafa et al., 2009).**

N-ethylmaleimide (NEM) is a sulfhydryl blocker that can be used experimentally to induce colon ulcers in the form of relatively constant lesions of a suitable size without any operation **(Satoh et al., 1997).**

Based on the aforementioned facts, the present investigation aims to investigate the effect of alcohol consumption on NEM-induced ulcerative colitis in experimental rats. To fulfil this purpose, colon GSH content as an oxidative stress biomarker, as well as colon MPO, TNF-α and NO_X contents as inflammatory biomarkers were measured, supported by colon macroscopic and microscopic examinations.

Material and methods:

Drugs:

N-ethylmaleimide (NEM) and ethanol were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Claycomb medium was obtained from LRH Biosciences, USA.

Ethanol (2.5 g/kg) was given orally for two weeks **(Iwaniec and Turner, 2013)**. The required dose for each 200 g rat was available in 1 ml of solution. According to the method of **Satoh et al. (1997),** 0.1 ml of 2% NEM (dissolved in 1% methylcellulose) was given via the intracolon route. All other chemicals for laboratory experimentation were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals:

Adult male Wistar rats weighting 160–200 gm were used. The animals were obtained from the Research Institute of Ophthalmology (Giza, Egypt). The animals were left to acclimatize for one week at the animal facility of the Faculty of Pharmacy, Beni-Suief University (Egypt), before subjecting them to experimentation. Animals were provided with a standard pellet diet and were kept at a temperature of 22 ± 3°C and a 12-hour light/dark cycle as well as a constant relative humidity throughout the experimental period. All procedures in this study were carried out according to guidelines of Ethics Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Experimental design:

Rats were allocated into 4 groups each consisting of 8-10 rats. Animals were divided into a normal control group which received distilled water orally, an ethanol group which received ethanol (2.5 g/kg, orally) once daily for two weeks (Iwaniec and Turner, 2013), an ulcerative colitis (UC) control group which received distilled water orally and single dose of 2% NEM via the intracolon route and an ethanol/UC group which received ethanol (2.5 g/kg, orally) once daily for two weeks and a single intracolon dose of 2% NEM.

Methodology:

Induction of UC:

On the 8th day of the experiment, induction of colitis was performed according to the method of **Satoh et al. (1997).** It is based on the fact that sulfhydryl (SH) blockers such as NEM cause injury to the mucosa through decreasing the amount of defensive SH compounds.

Rats were individually housed in metal cages with wide-meshed bottom to prevent coprophagy. The animals were fasted for 24 hours. Tested drug were administered orally one hour before induction of UC. Animals of UC and ethanol/UC groups were given intracolon 0.1 ml of 2% NEM dissolved in 1% methylcellulose intracolon at a depth of 6 cm from anus via a Nelaton's catheter. Normal control group was given 0.1ml of 1% methylcellulose only.

Twenty-four hours after the last day of the experiment, the rats were sacrificed by cervical dislocation. The colon was excised and the colitis was assessed macroscopically by measuring both colon length and the ratio of colon weight in milligrams to total body weight in grams which was taken as the colon mass index.

The colon was cut at a depth of 7 cm from the anus and opened longitudinally. One specimen was fixed in 10 % formalin solution in saline and preserved for histological examination, and the other was homogenized in ice-cold normal saline to obtain a 10 % homogenate for assessment of the chosen biochemical parameters.

Determination of biochemical parameters:

The colon homogenate was divided into four aliquots for determining the following parameters:

Reduced glutathione (GSH):

One aliquot was deproteinized with ice cold 5 % sulfosalicylic acid then centrifuged at 3,000 rpm for 20 min. The supernatant was used for spectrophotometric estimation of GSH according to the method of **Beutler et al., (1963).**

Total Nitrate/Nitrite (NO_X) production:

After protein precipitation with cold absolute ethanol, the supernatant was centrifuged for 40 minutes at 13,500 rpm at 4ºC using cooling centrifuge. The supernatant was used for spectrophotometric estimation of NO_X according to the method described by **Miranda et al. (2001).**

Myeloperoxidase activity (MPO):

An aliquot was mixed with an equal volume of 100 mmol/L phosphate buffer pH 6 containing 1 % hexadecyltrimethylammonium bromide. The mixture was freeze-thawed, sonicated for 10 sec and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was used for spectrophotometric estimation of MPO activity according to the method of **Krawisz et al. (1984)**

Tumor necrosis factor alpha (TNF)-α:

An aliquot was centrifuged at 13000 rpm for 30 minutes at 4°C. The supernatant was usedfor assaying TNF-α using enzyme-linked immunosorbent assay kits.

Histopathological examination:

The hardened colons were sectioned and embedded in paraffin then stained with hematoxyline and eosin according to the method described by **Bancroft and Gamble (2008)** and then examined under a light microscope.

Statistical analysis:

Data analysis was achieved using a software program Prism (version, 5). Data were presented as means ± S.E. Comparisons between different treatments were done using one way ANOVA followed by Student-Newman-Keuls as a post-ANOVA test. Criterion for significance was chosen to be at $p < 0.05$.

Table (1): Effect of ethanol consumption on colon length in normal rats and in rats with NEM-induced UC.

Parameters / Groups	Colon Length (cm)	
	Absolute values	**% of ulcer control**
Normal control group	**18.00** ± 0.54	**138.46** ± 4.15
Ethanol group	**16.00**[a] ± 0.25	**123.08**[a] ± 1.92
UC control group	**13.00**[a b] ± 0.48	**100.00**[a b] ± 3.69
Ethanol / UC group	**13.00**[a b] ± 0.18	**100.00**[a b] ± 1.38

Data are expressed as mean ± SEM (n=6-8). Multiple comparisons were done using one way ANOVA followed by Student- Newman-Keuls as post ANOVA test.
[a]Significantly different from normal control group at $p < 0.05$.
[b]Significantly different from ethanol group at $p < 0.05$.

ANOVA: Analysis of variance, NEM: N-ethylmaleimide, UC: Ulcerative colitis.

Table (2): Effect of ethanol consumption on colon mass index in normal rats and in rats with NEM-induced UC.

Parameters / Groups	Colon Mass Index (mg/g)	
	Absolute values	**% of ulcer control**
Normal control group	**09.30** ± 0.58	**71.54** ± 4.46
Ethanol group	**08.20** ± 0.35	**63.08** ± 2.69
UC control group	**13.00**[a b] ± 1.10	**100.00**[a b] ± 8.46
Ethanol / UC group	**12.00**[a b] ± 1.20	**92.31**[a b] ± 9.23

Data are expressed as mean ± SEM (n=6-8). Multiple comparisons were done using one way ANOVA followed by Student- Newman-Keuls as post ANOVA test.
[a]Significantly different from normal control group at $p < 0.05$.
[b]Significantly different from ethanol group at $p < 0.05$.

ANOVA: Analysis of variance, NEM: N-ethylmaleimide, UC: Ulcerative colitis.

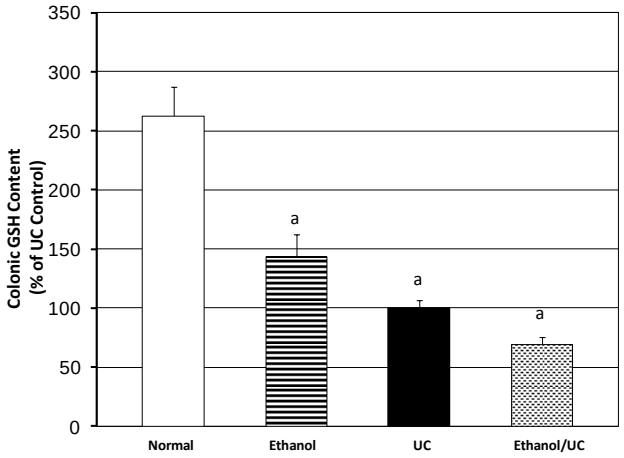


Figure (1): Effect of ethanol consumption on colonic GSH content in normal rats and in rats with NEM-induced UC.

Values represent means of 6-8 rats (expressed as % of UC control) ± SE. Multiple comparisons were done using one way ANOVA followed by Student- Newman-Keuls as post ANOVA test.

[a]Significantly different from normal control group at $p < 0.05$.

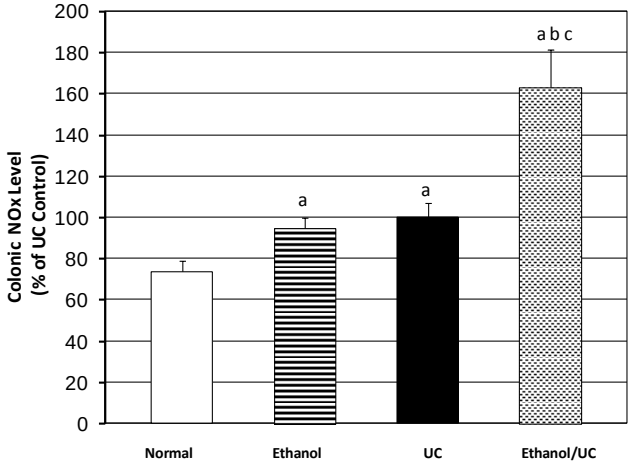


Figure (2): Effect of ethanol consumption on colonic total NO_x level in normal rats and in rats with NEM-induced UC.

Values represent means of 6-8 rats (expressed as % of UC control) ± SE. Multiple comparisons were done using one way ANOVA followed by Student- Newman-Keuls as post ANOVA test.
[a]Significantly different from normal control group at $p < 0.05$.
[b]Significantly different from ethanol group at $p < 0.05$.
[c]Significantly different from UC group at $p < 0.05$.

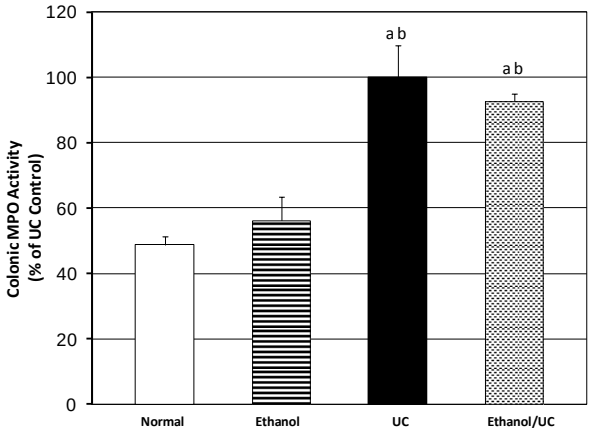
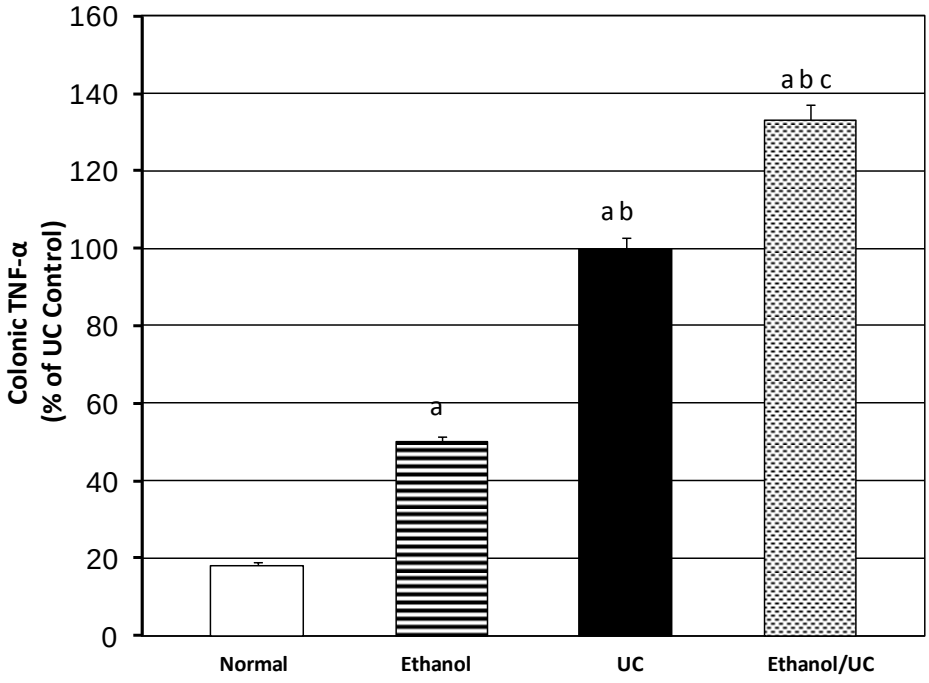


Figure (3): Effect of ethanol consumption on colonic MPO activity in normal rats and in rats with NEM-induced UC.



Values represent means of 6-8 rats (expressed as % of UC control) ± SE. Multiple comparisons were done using one way ANOVA followed by Student- Newman-Keuls as post ANOVA test.
[a]Significantly different from normal control group at $p < 0.05$.
[b]Significantly different from ethanol group at $p < 0.05$.

Figure (4): Effect of ethanol consumption on colonic TNF-α in normal rats and in rats with NEM-induced UC.

Values represent means of 6-8 rats (expressed as % of UC control) ± SE. Multiple comparisons were done using one way ANOVA followed by Student- Newman-Keuls as post ANOVA test.

[a]Significantly different from normal control group at $p < 0.05$.
[b]Significantly different from ethanol group at $p < 0.05$.
[c]Significantly different from UC group at $p < 0.05$.

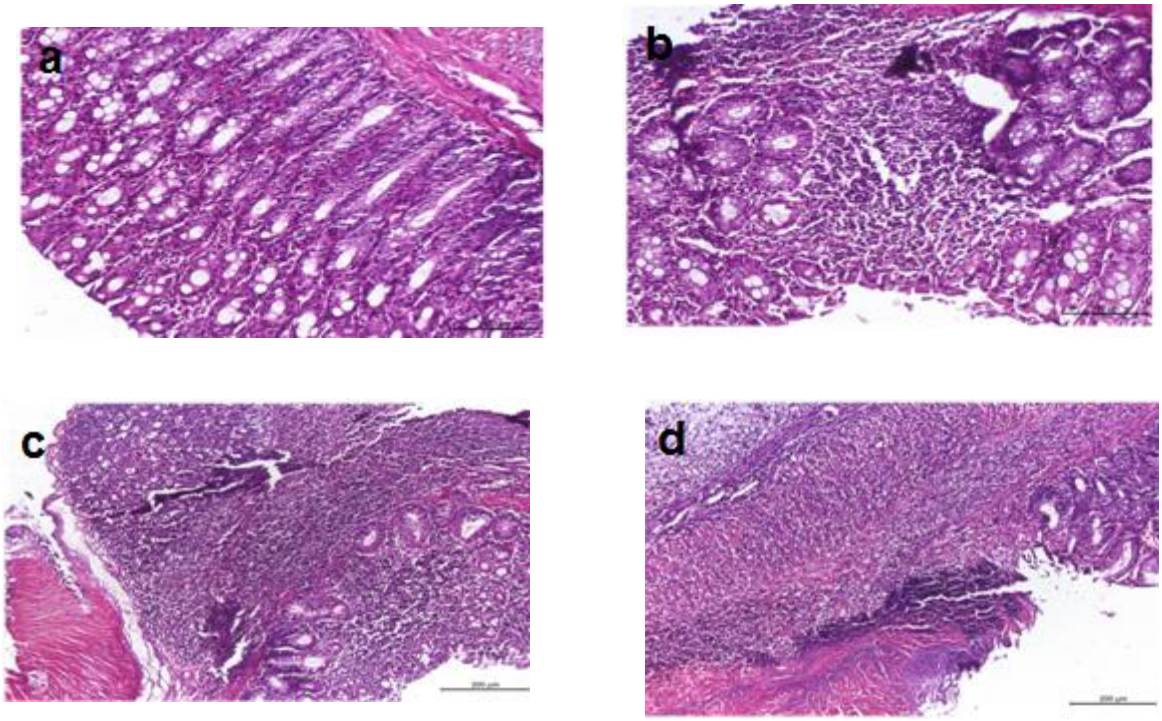


Figure 5: Effect of two weeks daily oral treatments with ethanol on histopathological changes of rat colon in a model of NEM-induced colitis.
a: A photomicrograph of colon section obtained from a normal control rat showing normal histological structure of mucosa; b: A colon section from the ethanol group showing mild ulcerative changes in the upper layers of the mucosa; c: A colon section from the NEM-treated group showing sever ulcerative changes with almost destruction of crypts; d: A colon section from the Ethanol/ UC group showing severe ulcerative changes with marked destruction of the mucosa. (H&E staining × 200).

Discussion

Ethanol use represents a serious challenge to public health that affects over 75 million people worldwide and accounts for 2.5 million deaths/year. The toxic by-products created when ethanol is broken down are carried through the bloodstream to just about every cell and tissue in the body **(Caan and de Belleroche, 2002).** Ethanol consumption has been associated with a wide variety of deleterious health effects such as liver disease and cardiomyopathy **(Klatsky, 2007).**

According to the results of the present investigation, ethanol decreased the colon length as compared to normal control rats (Table 1). In addition, administration of ethanol to UC control rats decreased the colon length and increased the colon mass index (Table 2). The increase in colon mass index may be related to the sub-mucosal oedema shown histologically while the colon shortening may be associated with colon thickening due to oedema and infiltration of inflammatory cells into the lamina propria and sub-mucosa **(Kraft and Kirsner, 1971)**. This

revealed that ethanol consumption led to increase in the inflammatory infiltrate in both lamina propria and sub-mucosa of normal colon.

Ethanol consumption reduces the levels of antioxidant enzymes as well as other antioxidants as GSH **(Colell et al., 1998; Nanji and Sturmhöfel, 1997).** This observation agrees with results of the present work that showed the depletion of the colonic GSH content after treatment of ethanol as compared to normal control rats (Figure 1). Similar findings were reported by **Macdonald et al. (2010)** who reported that the decrease of GSH is obviously connected with ethanol induced oxidative stress, which is characterized by the generation of toxic acetaldehyde and other reactive molecules in the cell. While, there was no significant increase in depletion of GSH after administration of ethanol to UC control rats as compared to UC control rats (Figure 1). This may be explained by the fact that NEM has strong effect on the depletion of GSH **(Satoh et al., 1997).** NEM blocks sulfhydryl groups that are crucial for the vitality of the intestine. GSH serves as the main pool of sulfhydryl groups and in its reduced form (GSH) protects epithelial cells from oxidative stress **(Kenet et al., 2001)**.

Results of the present study showed that ethanol increased the production of NO_x as compared to normal control rats (Figure 2). This finding is in accordance with results of **Lizarte et al. (2009)** and **Leite et al. (2013)** who reported that ethanol increases iNOS which produces massive amounts of NO **(Curran et al., 1989).** In addition, there was a massive increase in the production of NO_x after administration of ethanol to UC control rats as compared to UC control rats (Figure 2). According to **Albano et al. (1998)** and **Cederbaum (2001),** ethanol consumption did not only activate free radical generation, but also alters the levels of both enzymatic and nonenzymatic endogenous antioxidant systems. This results in oxidative stress which induces iNOS expression by activation of protein kinase C signaling pathway by ROS (**Duval et al., 1995; Bagchi et al., 1997).** It can be concluded from this results that ethanol consumption, even in moderate dose, is consider as a risk factor for UC.

Neutrophil infiltration is usually associated with increased myeloperoxidase (MPO) activity, a marker of oxidative process, which correlates well with the severity of the lesions in NEM induced-UC **(Satoh et al., 1997)**. This was indeed the case in the present study (Figure 3). Furthermore, MPO itself possesses cytokine-like properties and can activate neutrophils **(Lau et al., 2005**). An increase in MPO activity after administration of ethanol to UC control rats is therefore indicative of an induction of neutrophil infiltration and induction of the inflammatory process. While, treatment of ethanol in normal rats did not produce any increase in the MPO level as compared to normal control rats (Figure 3). This result might be due to the dose of ethanol (once daily) as **Oh et al. (2005**) reported that repeated ethanol consumption (twice daily) increases gastric MPO activation.

Tumor necrosis factor-alpha (TNF-α) exerts its effects partly through promoting the release of other pro-inflammatory mediators (IL-6 and IL-1β; **Begue et al., 2006)**. Both TNF-α and IL-1β have been implicated in stimulating the production of IL-8 **(MacDermott, 1996)**, a chemokine implicated in the pathophysiology of IBD by recruiting neutrophils to the intestinal mucosa **(Umehara et al., 2006)**. Depending on results of the present study, the level of TNF-α was significantly raised in the colon following induction of colitis by NEM (Figure 4). Furthermore, treatment of ethanol increased the level of TNF-α in colon as compared to normal control rats. The obtained results agree with the findings of **Afshar et al. (2015)** who studied the immunomodulatory effects of ethanol consumption (Figure 4). Moreover, administration of ethanol to UC control rats increased the rise in the TNF-α level compared to UC control value. The increase in TNF-α level by ethanol may be explained by the increase in liver Kupffer cell activity which is believed to play a major role in the regulation of pro-inflammatory mediators **(Khoruts et al., 1991; Schäfer et al., 1995; Nakamura et al,. 2004).**

In conclusion, ethanol consumption even in moderate doses has harmful effects on normal colon and worsens UC disease progression, probable through decreased endogenous antioxidant capacity with increased inflammatory progression.

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