Original Article

Diazepam dose-dependently Aggravates Mucosal Damage in a Rat Model of Ulcerative Colitis

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A B S T R A C T

BACKGROUND: Psychiatric co-morbidities, such as anxiety, are common in patients with chronic gut disorders, including those with overt inflammatory conditions of the gastrointestinal tract. Among the available pharmacological options, anxiolytics such as benzodiazepines (BZDs) are considered to be effective drugs; however, no sufficient data are available about their direct effect on gut mucosal impairment.

OBJECTIVES: The present work was designed to examine the effect of different doses of diazepam on a rat model of ulcerative colitis.

METHODS: Three doses of diazepam were assessed comparatively in an experimental model of ulcerative colitis. Thirty six male albino rats were divided into 5 groups, each consisting of 6 animals; Group I: Sham operated group, Group II: ulcerative colitis was induced with 1 ml 5 % acetic acid by intracolonic instillation without treatment. Groups III, IV&V: diazepam 1, 3 or 6 mg/kg respectively, was administered i.p. for two days with and after inducing ulcerative colitis. Group VI: Vehicle treated group received polyethylene glycol i.p. Distal colon segment was evaluated both macroscopically and microscopically for the degree of damage. The inflammatory response was assessed by measurement of colonic myeloperoxidase activity (MPO), serum tumor necrosis factor (TNF)-α, as well as fecal lactoferrin and calprotectin levels.

RESULTS: Rats with induced colitis showed macroscopic & microscopic signs of inflammation; this was associated with a significant increase in TNF-α, MPO as well as fecal lactoferrin and calprotectin contents. In a dose-dependent manner, diazepam administration, at the 3 dose levels, exacerbated the damage produced by acetic acid, this was revealed by gross inspection, as well as histologically and biochemically.

CONCLUSIONS: Accordingly, the current study displayed that diazepam has deleterious effects when administrated to colitic rats.

Key Words: Diazepam, Ulcerative Colitis, Acetic acid, TNF-α, MPO, Lactoferrin, Calprotectin, rats.

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1. INTRODUCTION

Anxiety is a frequent comorbidity in gut disorders, including inflammatory bowel disease (IBD) (*Sajadinejad et al., 2012*). The focus on chronic inflammation in previous studies provided support for a bidirectional relationship between behavior and gut inflammation; anxiety increases vulnerability to gut inflammation, which, in turn, induces anxiety-like behavior and alters central nervous system biochemistry, which can be normalized by inflammation-dependent and -independent mechanisms (*Bercik et al., 2010*).

Sajadinejad et al. (2012) identified mechanisms of the effects of psychological stress on patients with IBD as non-specific and immunological factors. Stimulation of corticotrophin releasing factor (CRF) (Mawdsley and Rampton, 2005) and release of mediators such as eicosanoids, serotonin and interleukin-6 (IL-6) inhibit the upper gastrointestinal motility & stimulates the colonic motility and increases intestinal paracellular permeability via mast cell dependent release of tumor necrosis factor α (TNF-α) (Tacheé et al.,2009). **Moreover, Knowles et al. (2013)** showed that anxiety produced 30% exaggerated symptoms of IBD. Emerging trial evidence supports the suggestion that psychologically

oriented therapy may ameliorate IBD-associated mood disorders (***Goodhand et al., 2009***).

On the other hand, cytokine-induced sickness behavior is a well-recognized entity that comprises neurovegetative and psychological factors, including depression and anxiety (***Bercik et al., 2010).***

Treatment of colitis has to consider the natural history of colonic inflammation. Colitis seems to have two phases; early phase with the influence of oxygen radicals and reactive oxygen species (***Tannahill et al.,1995)*** and the late phase which occurs after hours when T cell activation , proliferation and production of proinflammatory mediators take place (***Ostanin et al., 2012)***. The infiltrated neutrophils represent an important source of reactive oxygen and nitrogen species inducing cellular oxidative stress (***Mustafa et al., 2006)***.

Accordingly, myeloperoxidase (MPO) activity has been used as an indicator of neutrophil influx into inflamed gastrointestinal tissue (Mustafa et al., 2006). ***Krawitz et al. (1984)*** considered the measurement of MPO and TNF-α, as quantitative measures of disease severity and a method of assessing drug efficacy in animal models of intestinal inflammation. In addition, ***Sugi et al. (1996) and*** **D'Incà** *et al. (2007)* verified that both calprotectin and lactoferrin tests are useful in detecting bowel inflammation in symptomatic patients. Lactoferrin is one of the components of the immune system of the body; it has antimicrobial activity and is part of the innate defense, mainly at mucosal surfaces (Serrano-Luna et al., 2013). Fecal lactoferrin is 90% specific for identifying inflammation in patients with active ulcerative colitis (UC) and 100% specific in ruling out IBS (***Langhorst et.al.2005).***

Among the available pharmacological options, anxiolytics such as benzodiazepines (BZDs) are easily administered and show excellent safety and tolerability (***Olkkola and Ahonen, 2008)***. GABA receptors for BZDs are also located in the gut, stimulation of which depresses gastrointestinal movement and this may explain why the gut naturally produces BZDs, to keep the natural state of calm that is necessary for proper functioning (***Salari and Abdollahi, 2011)***. Accordingly, we designed our work to examine the effect of diazepam in modulating histopathological changes, oxidative stress and inflammation induced by acetic acid in an ulcerative colitis model in rats.

2. MATERIALS AND METHODS

2.1. Chemicals

Diazepam ampoules (10mg/2ml) (dissolved in polyethylene glycol) was purchased from Memphis Co. for Pharm. and Chemical Ind. (Cairo, Egypt) and acetic acid 96% was purchased from Sigma-Aldrich Chemical Co. (MO, U.S.A).

2.2 Animals

All animal procedures were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine. Thirty six male Wistar rats (weighing 150 to 200 g) purchased from National Research Institute (Cairo, Egypt) were housed at a temperature of 22-24 °C, relative humidity 50–70%, and 12 h light–dark cycle. An adaptation period of 1 week was allowed before initiation of the experimental protocol.

2.3 Experimental Procedure

Twelve hours before the intracolonic instillation of acetic acid or saline, feeding was stopped and the rats were only allowed to drink water.

Experimental groups: the rats were subdivided into six groups (6 rats each):

Group I: sham operated group; rats in head-down position received 1ml saline solution followed by 2 ml of phosphate buffer solution (pH: 7) after 30 seconds using soft pediatric catheter for intracolonic instillation at 8 cm proximal to the anus under ether anesthesia. The rats were maintained in this position for another 30 seconds to prevent leakage, and the rest of the solution was aspirated (***Mustafa et al., 2006)***.

Group II: control ulcerative colitis group; one ml of 5% acetic acid solution (pH: 2.4) diluted in distilled water was slowly administered followed by the phosphate buffered solution after 30 seconds using soft pediatric catheter for intracolonic instillation under ether anesthesia (***Sakurai et al.,1998).*** Colonic injury was apparent after 24 hours.

Group III, IV and V; diazepam treatment, 1, 3 or 6 mg kg[1]day[1] i.p. respectively (***Musavi and Kakkar, 1998),*** was administered to the rats with induction of ulcerative colitis and after 24 hours.

Group VI; vehicle-treated group; rats received polyethylene glycol that was given i.p.

2.4. Collection of the samples

Retro-orbital blood samples were collected 48 hours after the intracolonic instillation from all rats and allowed to clot for separation of serum used in the biochemical assays.

Then, all the rats were sacrificed using deep ether anesthesia. Laparotomy was done and a 10 cm colon segment was then dissected totally through proximal end. During this dissection, the feces in the colon were purified by hand caressing and dried after

washing with saline for detection of lactoferrin and calprotectin. The proximal 5 cm was scored macroscopically and maintained in formalin for microscopic studies. The distal 5 cm were taken and homogenized for assessing myeloperoxidase (MPO) activity.

2.5. Assessment of ulcerative colitis

2.5.1. Grading and Macroscopic Scoring

According to *Millar et al. (1996)*, the colonic mucosa was scored by an independent observer according to a scale ranging from 0 to 4 as follows:

Grade 0: Normal mucosal pattern
Grade 1: Scattered erosions
Grade 2: Linear ulcerations
Grade 3: Diffuse inflammatory tissue featuring small lesions of less than 5 mm
Grade 4: Diffuse ulcerations, wide lesions .

2.5.2. Histopathological study

Full thickness biopsy specimens were fixed in 10% formalin prior to wax embedding, sectioning and staining with haematoxylin and eosin for histological evaluation of colonic damage by light microscopy (X 100 and X 200).

2.6. Determination of MPO

This was performed according to *Bradley et al. (1982)*. The principle of the method depends on release of MPO enzyme in the homogenate of the colonic tissue used. Its level was detected using 0.3 mmol of H_2O_2 as a substrate. A unit of MPO activity is defined as that converting 1 _mol of H_2O_2 to water in 1 min at 25 ∘C. In brief, segments of the distal colon (0.5 g) were homogenized in 10 vol. of 50 mM sodium phosphate buffer (pH 7.4) in an ice-bath. The pellet (containing 95% of the total tissue MPO activity) was resuspended in an equal volume of potassium phosphate buffer (pH 6). Another centrifugation step for a period of 20 min at $16,000 \times g$ was done. The resultant supernatant was used for MPO assay using tetramethylbenzidine (TMB). The activity of MPO was measured using spectrophotometer. Reagents for MPO assay were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A) and measured according to manufacturer's instructions.

2.7. Assay of fecal calprotectin and lactoferrin

Fecal samples obtained from rectum were weighed, homogenized in buffer solution, and centrifuged, calprotectin *(Dief et al.,2008)* and lactoferrin *(Togawa et al.,2002)* in the supernatants were measured by ELISA kits (Buhlmann calprotectin ELISA Kit, Schönenbuch, Switzerland) and (EIAab

Science Lactoferrin, ELISA Kit, Wuhan, China), respectively according to manufacturer instructions**.**

2.8. Measurement of serum TNF-α activity

Serum TNF-α levels were measured by rat ELISA kits (ALPCO Diagnostics ELISA kit, NH, USA) according to manufacturer instructions.

2.9. Statistical analysis

Statistical analysis was carried out using Graph pad prism, software program, version 5.0 (2007), Inc., CA, USA. All values were expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) for comparison of the different groups was used followed by Tukey post hoc test for multiple comparisons between groups with significance set at p < 0.05. A non-parametric way of analysis was used to assess the median of macroscopic tissue injury**.**

3. RESULTS

The overall evaluation of the results revealed that the rats of the sham operated and vehicle treated groups showed no alternations after macroscopic, microscopic and biochemical assessments**.**

3.1. Effect of diazepam on macroscopic scoring

The acetic acid treatment induced macroscopic inflammation in the colon 24 h after rectal administration, as assessed by the colonic damage score reached. Diazepam (1, 3 or 6 mg/ kg) significantly increased the severity of the gross lesion scores in a dose dependent manner (Table 1 and Fig. 1 A-E) with mean % values of 50 & 68 and 128% respectively. Moreover, the median, minimum and maximum response increased gradually from 2&1&2 of group II to 4&3&4 in group VI.

3.2. Effect of diazepam on microscopic alterations induced by acetic acid

The histopathological features of acetic acid treated rats showed massive edema separating the glands. Rats treated with diazepam showed congestion, edema and diffuse inflammatory cell infiltration in the mucosa with transmural necrosis. There was focal ulceration of the colonic mucosa extending through the muscularis mucosa & desquamated areas. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts especially in basal areas. An infiltrate consisting of mixed inflammatory cells was observed (Fig.2 A-E)

3.3. Effect of diazepam on MPO in acetic acid treated rats

Table (2) shows that, MPO activity increased significantly with acetic acid instillation. Diazepam

administration induced significant increase in the MPO activity as compared to acetic acid treated group. MPO increased from 1.15+0.05 to 1.99+0.16 & 3.9+0.21 and 4.96+0.33 in groups III, IV and V respectively.

3.4. Effect of diazepam on Fecal Lactoferrin and Calprotecin in acetic acid treated rats:

As shown in Table (3), fecal lactoferrin and calprotecin significantly increased with acetic acid instillation (group II). Diazepam treatment induced significant increase in both fecal parameters. Lactoferrin and calprotecin increased from 53.62+3.57

and2.78+0.28 in group II to 114.4+2.98 and 5.37+0.57 & 208.7+4.26 and 9.08+0.93 & 223.7+5.62 and 10.75+0.92 in groups III, IV and V respectively.

3.5. Effect of diazepam on serum tumor necrosis factor-α (TNF-α)

Table (4) shows that TNF-α increased significantly with acetic acid installation (group II). Diazepam increased the inflammation significantly as indicated by increase TNF-α from 65.02+5.73 of group II to 99.63+3.59&160.2+8.26 and 177.2+9.24 in groups III, IV and V respectively.

Table (1): Effects of different doses of Diazepam (1, 3 and 6 mg kg^{-1}) on gross lesion score of acetic acid-induced ulcerative colitis in rats

Group	Treatment	Gross lesion	Median	Minimum	Maximum	% Change
I	Sham group	00+0.00	0	0	0	-
II	Acetic acid (5%)	1.67+0.52	2	1	2	-
III	Diazepam 1 mg Kg^{-1}	2.5+0.84	3	1	3	+50% #
IV	Diazepam 3 mg Kg^{-1}	2.8+0.75	3	2	4	+68% #
V	Diazepam 6 mg Kg^{-1}	3.8+0.41	4	3	4	+128% #

Values are expressed as mean ± S.D. (n=6), median, minimum and maximum response. The percentage change indicates the change of colonic lesion as compared to acetic acid group. A non-parametric way of analysis was used to assess the median of macroscopic tissue injury.
#$P < 0.05$ in comparison to acetic acid group

Table (2): Effects of different doses of Diazepam (1, 3 and 6 mg kg^{-1}) on myeloperoxidase (MPO) of acetic acid-induced ulcerative colitis in rats

Groups	Treatment	MPO (mean+SD) U/g tissue
I	Sham group	0.82+0.04
II	Acetic acid 5%	1.15+0.05*
III	Diazepam 1 mg kg^{-1}	1.99+0.16*#
IV	Diazepam 3 mg kg^{-1}	3.9+0.21*#
V	Diazepam 6 mg kg^{-1}	4.96+0.33*#

The values are expressed as mean ± S.D. (n=6). Analysis of variance (ANOVA) for comparison of the different groups was used followed by a Tukey post hoc test for multiple comparisons between groups.
*P<0.05 in comparison to sham
#$P < 0.05$ in comparison to acetic acid group

Table (3): Effects of different doses of Diazepam (1, 3 and 6 mg kg⁻¹) on fecal lactoferrin and calprotectin of acetic acid-induced ulcerative colitis in rats

Groups	Treatment	Fecal Lactoferrin (ng/ml)	Fecal Calprotectin (ng/ml)
I	Sham	39.52+0.55	1.41+1.16
II	Acetic acid 5%	53.62+3.57	2.78+0.28*
III	Diazepam 1 mg kg⁻¹	114.4+2.98	5.37+0.57*#
IV	Diazepam 3 mg kg⁻¹	208.7+4.26	9.08+0.93*#
V	Diazepam 6 mg kg⁻¹	223.7+5.62	10.75+0.92*#

Values are expressed as mean ± S.D. of six animals per group. Analysis of variance (ANOVA) for comparison of the different groups was used followed by a Tukey post hoc test for multiple comparisons between groups
*P<0.05 in comparison to Sham
#*P* < 0.05 in comparison to acetic acid group

Table (4): Effects of different doses of Diazepam (1, 3 and 6 mg kg⁻¹) on TNF-α of acetic acid-induced ulcerative colitis in rats

Groups	Treatment	TNF-α (pg/ml)
I	Sham	38.77+2.64
II	Acetic acid 5%	65.02+5.73*
III	Diazepam 1 mg kg⁻¹	99.63+3,59*#
IV	Diazepam 3 mg kg⁻¹	160.2+8.26*#
V	Diazepam 6 mg kg⁻¹	177.2+9.24*#

Values are expressed as mean ± S.D. of six animals per group. Analysis of variance (ANOVA) for comparison of the different groups was used followed by a Tukey post hoc test for multiple comparisons between groups.
*P<0.05 in comparison to sham
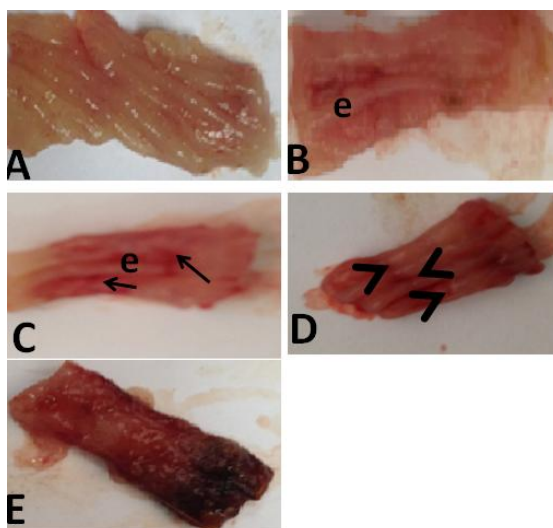#*P* < 0.05 in comparison to acetic acid group

Figure (1,A-E); Gross macroscopic pictures showing the effect of two intraperitoneal injections of diazepam one injection with intracolonic inistillation of acetic acid and one after one day of instillation on the colonic surface. Segments were taken from: A; sham group ,B; acetic acid (5% 1 ml+ phosphate buffer 2ml PH 7 intracolonic inistillation) group,(C-E); Diazepam (1, 3 or 6 mg/ kg⁾ groups respectively.
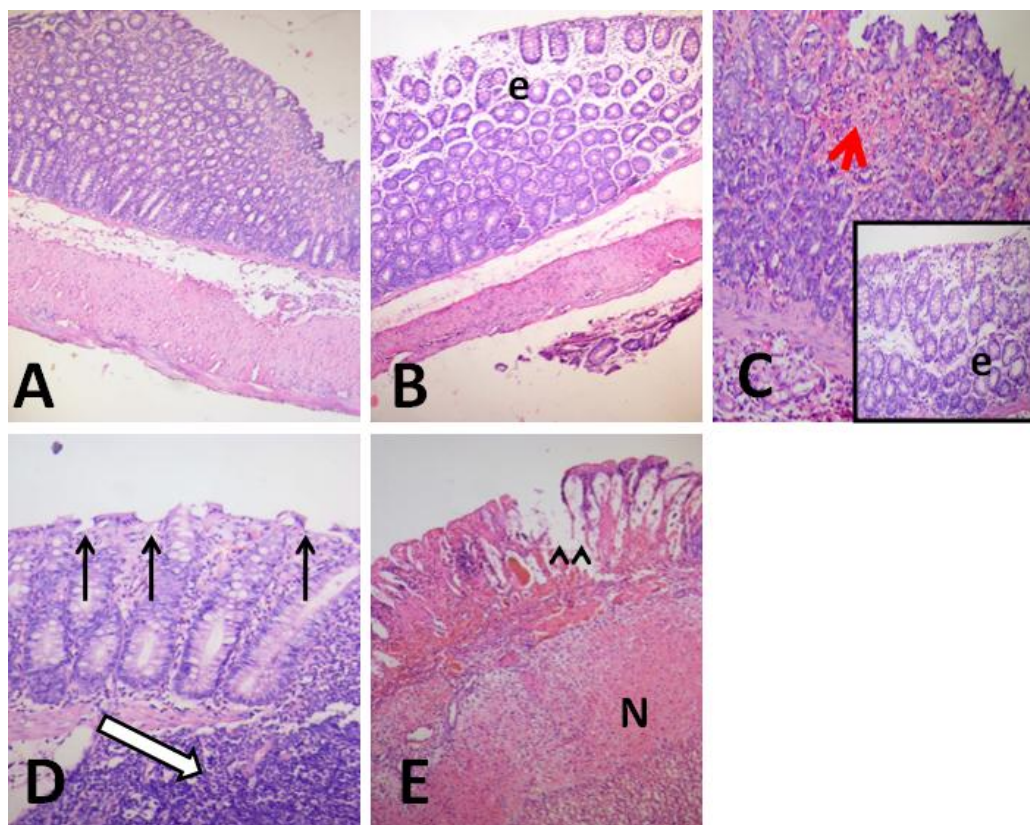[e: edema, ↑ congestion , ▲ surface erosion]

Figure (2,A-E); Photomicrograph of the haematoxylin and eosin stained section of rat colon X 100 (the inset X200) showing the effects of two intraperitoneal injections of diazepam one injection with intracolonic inistillation of acetic acid and one after one day of instillation. Segments were taken from: A; sham group ,B; acetic acid (5% 1 ml+ phosphate buffer 2ml PH 7 intracolonic inistillation) group,(C-E); Diazepam (1, 3 or 6 mg/ kg) groups respectively.

[e: edema, : ∧ congestion , ↑surface erosion, ⇑massive invasion of inflammatory cells, ∧ ulceration, N: necrosis]

4. DISCUSSION

The rationale for the use of anxiolytic drugs for the treatment of inflammatory bowel diseases (IBDs) likely stems from the clinical observation that the majority of patients also show evidence of co-morbid psychological symptoms, particularly anxiety (Mayer et al., 2006). Benzodiazepine (BZD) receptors in both central and peripheral forms and their ligands create a regulatory network between anxiety and the immune system (Salari and Abdollahi, 2011)

The present study demonstrated that treatment of rats (exposed to colonic injury) with diazepam increased the inflammation and the acute colonic damage induced by acetic acid. While, acetic acid induced edema and congestion in the intestinal mucosa, diazepam induced edema, hemorrhage,

erosion and ulceration in some areas as was seen by the naked eye and verified under the microscope. Moreover, acetic acid caused an increase in MPO activity, TNFα and both fecal lactoferrin and calptotecin. All these changes were further enhanced with diazepam in a dose-dependent way.

The effect of acetic acid on the colon was investigated before by Kanodia et al. (2011) who studied the effect of fruit extract of *Fragaria vesca L.* on acetic acid induced ulcerative colitis (UC) in albino rats and they provided results inconsistent with ours. While, Krawitz et al.(1984) considered the measurement of MPO and TNF-α a quantitative measure of disease severity and a method of assessing drug efficacy in animal models of intestinal inflammation, Sugi et al. (1996) and D'Incà et al.(2007) verified that both calprotectin and lactoferrin

tests are useful in detecting bowel inflammation in symptomatic cases.

In the last few years, the effect of BZDs on the immune system and inflammation was studied. Obiora et al. (2012) found that BZDs are associated with an increased risk of, and mortality from, community acquired pneumonia. Abed et al. (2013), however, demonstrated that diazepam pretreatment exhibited anti-inflammatory property in cerulein induced acute pancreatitis in rats which is in accordance with the findings of Lazzarini et al. (2010) who showed that diazepam decreased the interaction of leukocytes with endothelial cells.

The previous data suggest that further research of the immune safety profile of BZDs is required. Evidence for a direct immunomodulatory action for BZD emerged from studies that demonstrated the presence of peripheral or mitochondrial BZD receptor, also referred to as the translocator protein (TSPO), on immune/inflammatory cells such as macrophages, neutrophils, leukocytes, and lymphocytes and may have a crucial role in the relationship between the CNS, behavior and immunity (Kam et al.,2012)

Covelli et al. (1991, 1998) approved that diazepam is inhibitory *in vivo* and *in vitro* for the phagocytic functions and antibody synthesis, its action being mediated via TSPO on immunocompetent cells. However, Mediratta and Sharma (2002) disapproved these findings and mentioned that long term administration of diazepam reversed the immunosuppressive effects of restrained stress.

de Lima et al. (2010) solved this argument. They analyzed the effects of diazepam on rat lymphocyte parameters, specifically on phenotype, cell proliferation and cell death. The effects of both acute and long-term (21 days) diazepam (1 and 10 mg/kg/day) administrations were evaluated. They found that the effects of diazepam differ by administering different doses of diazepam. Small dose of diazepam (1mg/kg) was found to act on GABA-A receptors and caused no change in immune parameters and unexpectedly, increases serum corticosteroid. On the other hand, de Lima and his colleagues mentioned that large doses of diazepam (10mg/kg) may have a direct immunomodulatory action, depending on the number of TSPO sites on immune cells. It increases the number of T lymphocytes, superoxide anion, hydroxyl radicals, oxo-ferry radical species and decreased the number of apoptotic cells.

The results presented by Ostuni et al. (2010) who studied the TSPO expression in IBD induced in rats via treatment with dextran sodium sulfate (DSS), demonstrated that TSPO is over expressed during early stages of inflammation in IBD and that may be the mechanism by which diazepam exaggerated the colonic inflammation in our work.

From the above, and taking together that UC is an immune related disease with an emotional inclination and diazepam presents an indefinite immunological effect, patients with IBDs are advised to avoid diazepam due to an unexpected immune response as demonstrated in our work. Interestingly, FDA reported 21 cases of colitis associated with diazepam administration in (2012) in a total of 29,872 diazepam users

http://www.ehealthme.com/ds/diazepam/colitis+ulcerative).

5. CONCLUSION

The data collected in this study suggested that the deleterious effects of diazepam on the colonic mucosa may be mediated by acting on another pathway rather than GABA-A receptor. To investigate the role and involvement of TSPO and/or GABA receptors, additional experiments using both in vitro and in vivo models is recommended.

6. ACKNOWLEDGEMENT

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