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Anxiety- and Depressive-Like Behaviors are Associated with Altered Hippocampal Energy and Inflammatory Status in a Mouse Model of Crohn's Disease

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Abstract—Depression and anxiety are common comorbid disorders observed in patients with inflammatory bowel disease (IBD). Increasing line of evidence indicates that immune-inflammatory responses are involved in co-occurrence of mood disorders and IBD. However, the mechanisms through which immune-inflammatory pathways modulate this comorbidity are not yet understood. This study investigated the role of innate immunity in the development of behavioral abnormalities associated with an animal model of Crohn's disease (CD). To do this, we induced colitis in male adult mice by intrarectal (i.r.) injection of DNBS (Dinitrobenzene sulfonic acid). After 3 days, we performed behavioral tests for anxiety- and depressive-like behaviors as well as tissue collection. Our results showed that DNBS-induced colonic inflammatory responses were accompanied by infiltration of inflammatory cells, and increased expression of genes involved in toll-like receptor signaling pathway in intestinal tissue. Furthermore, the DNBS-treated mice showed depressive- and anxiety-like behaviors which were associated with increased expression of the inflammatory genes and abnormal mitochondrial function in the hippocampus. These results suggest that peripheral inflammation is able to increase the transcriptional level of the genes in toll-like receptor pathway, induces abnormal mitochondrial function in the hippocampus, and these negative effects may be involved in the co-occurrence of anxiety and depression in early stages of CD. © 2017 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: depression, anxiety, Crohn's disease, oxidative stress, hippocampus, toll-like receptor pathway.

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a chronic disease associated with psychiatric comorbidities such as depression and anxiety (Chauhan et al., 2016; Mikocka-Walus et al., 2016). Psychiatric comorbidities in IBD patients negatively affect the quality of their lives and the severity of disease in sufferings (van den Brink et al., 2016; Yanartas et al., 2016). Evidence has shown that the incidence of mood disorders is higher in patients with IBD in comparison with other chronic diseases (Taft and Keefer, 2016). Recent preclinical investigations have revealed that induction of experimental colitis in rodents is able to provoke psychopathologies such as depressive- and anxiety-like behaviors in animals (Bercik et al., 2010). In this regard, it has been shown that dextran

sulfate sodium (DSS)-induced colitis in rodents is accompanied by behavioral comorbidities (Reichmann et al., 2015; Nyuyki-Dufe et al., 2016). Also, interleukin-10 knockout (IL-10−/−) mouse (an animal model for studying IBD) shows anxiety-like behaviors and memory impairments (Kühn et al., 1993; Ohland et al., 2013). Considering direct association between IBD and psychological disorders, only few studies have investigated the underlying mechanisms involved in the comorbidity of IBD and psychiatric disorders.

Emerging line of research suggests that oxidative and nitrosative stress (O&NS) and immune-inflammatory responses play important roles in the pathophysiology of depression (Maes, 2008; Maes et al., 2009, 2011b; Patki et al., 2013). Focusing on IBD, evidence has recently suggested that oxidative challenge and pro-inflammatory cytokines contribute to the pathophysiology of IBD (Ghia et al., 2009; Maes et al., 2011b; Triantafillidis et al., 2013). Evidence also suggests that peripheral inflammation in patients with chronic diseases is able to initiate the development of mood disorders by triggering inflammatory signaling in the brain (Taché and Bernstein, 2009; Bonaz and Bernstein, 2013). Several studies have suggested that gut damage is accompanied by the activation of peripheral inflammatory responses, which may be related to the development of mood disorders in IBD patients (Fleshner, 2013; McCusker and Kelley, 2013; Singhal et al., 2014). Toll-like receptors (TLRs) are the main components of innate immunity, which recognize the pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and damage-associated molecular patterns (DAMPs). Activation of TLRs leads to production of pro-inflammatory cytokines such as IL-1β and IL-6, and nitric oxide (NO) in the brain (Barksby et al., 2007; Klune et al., 2008; Diacovich and Gorvel, 2010; Fleshner, 2013). The elevated levels of pro-inflammatory cytokines and nitrosative stress are capable of inhibiting the mitochondrial respiratory chain components and result in cellular energy deficiency (Mancuso et al., 2007; Maes et al., 2011a; Morris and Maes, 2014). Considering the crucial role of mitochondria in massive production of reactive oxygen species (ROS) under pathological conditions, a number of studies suggest that abnormal mitochondrial function and inflammatory responses may contribute to the pathogenesis of depression (Rezin et al., 2009; Bakunina et al., 2015; Crupi and Cuzzocrea, 2016).

In this study, we tested whether (1) experimental model of Crohn's disease is associated with the development of behaviors related to anxiety and depression, and (2) peripheral inflammation following induction of colitis in mice activates the genes relevant to innate immunity and oxidative stress in the hippocampus.

EXPERIMENTAL PROCEDURES

Animals

We used male NMRI mice purchased from the Pasteur Institute, Tehran, Iran. All animals, weighing 25–30 g, were housed in the groups (4 mice in each cage), and were kept at the temperature of 21–23 °C under a 12-h consistent light/dark cycle and were given access to food and water ad lib. All tests were performed between 10:00 and 14:00 h. All procedures were done in line with the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23, revised 1978) and the institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences). All experimental groups have been demonstrated and designated in Table 1. Also, full efforts were made to minimize the use of animals and to optimize their comfort.

Induction and characterization of colitis

Induction of colitis. DNBS (Dinitrobenzene sulfonic acid) (Sigma) was used to induce colitis. For this purpose, 6 mg of DNBS dissolved in 100 μL of 50% ethanol and slowly inject intra-rectally for each mouse. Animals were deprived of food 24 h prior to colitis induction, and were anesthetized using Isoflurane inhalation (Sigma). DNBS was injected intra-rectally (i.r.) using a flexible catheter 5 cm in length. After that the mice were held upside down in a 45° position for 2 min in order to avoid leakage of the DNBS solution, and were returned to their home cages. Animals were assessed for the behavioral or molecular assessments 3 d after colitis induction when the peak of acute inflammation occurs (Hollenbach et al., 2005). Control groups received 100μL normal saline (i.r.) and were evaluated 3 d after injection.

Macroscopic assessment. Animals were euthanized under deep anesthesia using isoflurane inhalation (3 d after DNBS or saline injection), colon was dissected out and cut open longitudinally and gently cleaned using PBS. The assessment of inflammation was scored based on ulceration, inflammation, and extent of disease. The scoring system subordinates from following scale from 0 to 9: 0 = normal aspect of the mucosa, 1 = localized hyperemia without ulcerations, 2 = ulceration, 3 = ulceration with thickening of bowel wall at one site, 4 = two or more sites of ulceration and thickening of the bowel wall, 5 = major sites of damage extending < 2 cm along the length of the colon, and 6–9 = damage extending > 2 cm (with the score increasing by 1 for each centimeter of damaged tissue) (El-Salhy et al., 2014).

Histopathological evaluation. In order to microscopic assessments, the colon was cut into pieces and fixed in 10% formalin. Formalin-fixed colon segments were paraffin-embedded and cut into 5-μm divisions. Nine sections obtained from each colon and were deparaffinized using xylene and stained with hematoxylin and eosin (H&E) (Hasanvand et al., 2016). Histological analysis was done under light microscopy by a pathologist in consistent with the previously described method (Obermeier et al., 1999). Each score characterized the mean of nine sections of each colon.

Table 1. Experimental groups of the study: Injections and number of animals in each experimental group for behavioral or molecular/histopathological tests were illustrated

Groups	Injections	Numb.	Behavioral tests	Molecular/Histological tests
IBD1	DNBS (6 mg, i.r.)	8	OFT & FST	–
IBD2	DNBS (6 mg, i.r.)	8	HBT & splash test	–
IBD3	DNBS (6 mg, i.r.)	8	EPM & TST	–
IBD4	DNBS (6 mg, i.r.)	8	SPT	–
IBD5	DNBS (6 mg, i.r.)	8	–	Colon macroscopic and histopathologic evaluations
IBD6	DNBS (6 mg, i.r.)	8	–	Colon MPO activity
IBD7	DNBS (6 mg, i.r.)	4	–	Colon Gene expression
IBD8	DNBS (6 mg, i.r.)	4	–	HIPP Mitochondrial factors
IBD9	DNBS (6 mg, i.r.)	4	–	HIPP Nitrite assay
IBD10	DNBS (6 mg, i.r.)	4	–	HIPP inflammatory genes
Control1	Saline (100 μL, i.r.)	8	OFT & FST	–
Control1	Saline (100 μL, i.r.)	8	OFT & FST	–
Control2	Saline (100 μL, i.r.)	8	HBT & splash test	–
Control3	Saline (100 μL, i.r.)	8	EPM & TST	–
Control4	Saline (100 μL, i.r.)	8	SPT	–
Control5	Saline (100 μL, i.r.)	8	–	Colon macroscopic and histopathologic evaluations
Control6	Saline (100 μL, i.r.)	8	–	Colon MPO activity
Control7	Saline (100 μL, i.r.)	4	–	Colon Gene expression
Control8	Saline (100 μL, i.r.)	4	–	HIPP Mitochondrial factors
Control9	Saline (100 μL, i.r.)	4	–	HIPP Nitrite assay
Control10	Saline (100 μL, i.r.)	4	–	HIPP inflammatory genes

Histopathology was scored as follows: Epithelium (E): 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Infiltration (I): 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the L. submucosa. The total histological score represents the sum of the epithelium and infiltration score (total score = E + I).

Colonic myeloperoxidase (MPO) activity measurement. MPO was assessed based on previously described method (Abdolghaffari et al., 2010; Khanavi et al., 2014). In brief, bowel samples were crushed on ice and homogenized in 2 ml of 50 mmol/L phosphate buffer, pH 7.4. Homogenizer probe was placed in a container of ice. The homogenates were centrifuged for 30 min at 3500 g and 4 °C. The upper liquid was removed and 1 ml of 50 mmol/L phosphate buffer, pH 6 containing 0.5% Hexadecyltrimethylammonium bromide (Sigma) and 10 mmol EDTA (Sigma) added into sediment samples and sonicated and then centrifuged for 20 min at 12,000 g and 4 °C. Subsequently, 15 μl from upper liquid phase was added to an ELISA plate containing 240 μl of 50 mmol/L phosphate buffer, pH 6 containing 0.167 mg/ml o-dianisidine (Sigma) and 0.0005% hydrogen peroxide (Sigma, USA). The change in absorbance was measured spectrophotometrically at 460 nm for 3 min. One unit of MPO activity was described as the change in absorbance per min at room temperature in the final reaction and expressed as unit per mg protein of colon tissue.

Quantitative reverse transcription–PCR (qRT-PCR). RNA was extracted using Tripure isolation reagent (Roche) according to the company's instructions and quantified by a ND-100 spectrophotometer (Nanodrop Technologies). Changes in mRNA expression of desired genes were assessed by qRT–PCR after reverse transcription of 1 μg RNA from each sample with PrimeScript RT reagent kit (Takara) according to the manufacturer's order. The qRT–PCR was done on a light cycler apparatus (Roche Diagnostics) using SYBR Premix Ex Taq technology (Takara). Thermal cycling environment involved an initial activation phase for 30 s at 95 °C followed by 45 cycles including a denaturation step for 5 s at 95 °C and a combined annealing/ extension step for 20 s at 60 °C. In order to approve that whether all primers yield a single PCR product, melting curve analysis was applied. Hypoxanthine phosphoribosyl transferase1 (*Hprt1*), was considered as a normalizer and fold changes in expression of each target mRNA relative to *Hprt1* was calculated based on $2^{-\Delta\Delta Ct}$ relative expression formula as described earlier (Amini-Khoei et al., 2016; Amiri et al., 2017). The primer sequences are listed in Table 2.

Behavioral tests

Forced swimming test (FST). FST was used as an approved behavioral test for evaluating the despair behavior in which increase in the immobility time presents the depressive-like behavior (Porsolt et al., 1977; Cryan and Holmes, 2005). Mice were individually placed in an open glass cylinder (diameter: 10 cm, height: 25 cm) containing 19 cm water (23 ± 1 °C). Mice were permitted to swim for 6 min and the immobility time was documented during the last 4 min. Immobility behavior was measured when the animal remained floating motionless in the water and made only those necessary activities to keep its head above water.

Table 2. Primer sequences

| Gene | Sequence (5′ → 3′) | |
	Forward	Reverse
Bdnf	TTACCTGGATGCCGCAAACAT	TGACCCACTCGCTAATACTGTC
Il-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
Nlrp3	ATCAACAGGCGAGACCTCTG	GTCCTCCTGGCATACCATAGA
Hmgb1	GCTGACAAGGCTCGTTATGAA	CCTTTGATTTTGGGGCGGTA
Il-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
Tnf-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
Hprt1	TGCTCGAGATGTGATGAAGG	AAGCAGATGGCCACAGAACT
Myd88	ATCGCTGTTCTTGAACCCTCG	CTCACGGTCTAACAAGGCCAG
Tlr-2	CTCTTCAGCAAACGCTGTTCT	GGCGTCTCCCTCTATTGTATTG
Tlr-4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA

Tail suspension test (TST). In the TST, mouse was suspended on the edge of a rod 50 cm above a table top using adhesive scotch tape, located approximately 1 cm from the tip of the tail. Tail climbing was prohibited by passing the mouse's tail through a small plastic cylinder prior to suspension. The duration of immobility time was manually documented for 6 min. Mice were considered immobile only when they hung down passively and were completely immobile (Steru et al., 1985; Cryan et al., 2005).

Splash test. Splash test used to evaluate the motivational and self-care difficulties, in which grooming behavior of mice, is considered as an indirect measure of palatable solution drinking. A 10% sucrose solution was spewed on the dorsal coat of animals while they were in their home cages and mice were evaluated for 5 min. Duration of grooming activity behavior including nose/face cleaning, head washing, and body grooming was observed by a person blinded to the experiment (David et al., 2009; Marrocco et al., 2014; Haj-Mirzaian et al., 2015).

Sucrose preference test (SPT). SPT is considered as an approved test to evaluate the hedonic state of rodents (Wallace et al., 2009). In this regard, animals were introduced to two bottles of tap water, which were placed in the home cage of each mouse in the first two days (1st and 2nd days). Then, one of the bottles was substituted by a bottle having 1% sucrose solution for the second two days (3th and 4th days). On the test day (5th day), animals were deprived of food and water for 5 h and then sucrose preference was assessed in one hour of liquid consumption using two bottles of 1% sucrose solution and tap water (DNBS/saline were injected in the 2nd day). SPT was calculated using the following equation, which evaluates the fraction of 1% sucrose solution consumed to the total liquid consumed: Sucrose preference = Sucrose consumed/(Sucrose consumed + tap water consumed).

Open-field test (OFT). We used the OFT to elucidate the effects of colitis on motoric function and anxiety-like behaviors (Kulesskaya and Voikar, 2014; Amiri et al., 2016). The OFT apparatus consisted of a white opaque Plexiglas box measuring (50 cm × 50 cm × 30 cm) which was dimly illuminated. Animals were placed individually on the central zone of the apparatus (30 cm × 30 cm) and their behaviors were recorded by a camera for 5 min and were analyzed by Ethovision software version 8 (Noldus, Netherlands). Distance moved (horizontal activity), number of rearings (vertical activity) and time spent in the central zone were evaluated.

Hole-board test (HBT). Hole-board test presented as a reliable trial to assess the anxiety-like behaviors in the rodents (Takeda et al., 1998; Haj-Mirzaian et al., 2015). The apparatus was made of a white Plexiglas square (50 cm × 50 cm) with 16 equally sized holes (3 cm in diameter) and was located 50 cm above the floor. Mice were placed in the center of the board, and the number of head-dips was calculated in a 5-min period by an experimenter.

Elevated plus maze (EPM). The EPM is an appropriate test to assess the anxiety-like behavior in mice (Ducottet and Belzung, 2005). The apparatus was made of black opaque Plexiglas and consisted of two open (30 × 5 cm) and closed (30 × 5 × 15 cm) arms, which were attached by a platform area (5 × 5 cm). Testing room was dimly illuminated and animals were individually placed in the center of the EPM facing to closed arm and each behavioral session was videotaped for a 5-min period and was analyzed by Ethovision software version 8 (Noldus, Netherlands). The time spent in the open arms and number of entries into the open arms are described as percentages.

Nitrite assay

Mice in each group were fasted overnight and after scarification, hippocampi were quickly dissected out and soaked in the liquid nitrogen and kept at −80 °C freezer until the assays. To determine NO levels in the hippocampus, we measured nitrite levels as the result of the NO end product (Ding et al., 2010; Kordjazy et al., 2015). Nitrite levels were measured by a colorimetric assay based on the Griess reaction in each hippocampus sample. Concentration of nitrite was determined by reference to a standard curve of sodium nitrite (Sigma) and normalized to the weight of each sample.

Mitochondrial function

Animals were sacrificed and hippocampi were dissected out, washed with PBS and stored at $-80\,°C$. Tissue homogenization was done with cold mannitol solution (4 °C) containing 0.225 M D-mannitol, 75 mM sucrose and 0.2 mM EDTA. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4 °C. The supernatant was centrifuged at $10,000 \times g$ for 10 min as a source of hippocampal mitochondria. The heavy mitochondrial fraction was collected and re-suspended in the mannitol solution and, re-centrifuged twice at $12,000 \times g$ for 10 min. The resulting pellet (P_2 fraction), including both synaptic and non-synaptic mitochondria was re-suspended in desired buffer based on oxidative stress markers including ROS production, ATP, and glutathione (GSH) (Wieckowski et al., 2009). To ensure that the obtained suspension is pure mitochondria, MTT test was conducted to confirm the work according to previous works (Hosseini et al., 2014). Mitochondrial protein concentration was determined by the Coomassie blue protein-binding method using BSA as the standard (Bradford, 1976). To keep the uniformity of experimental condition, the mitochondrial samples (100 μg/ml mitochondrial protein) were used in all experiments.

ROS formation. To measure mitochondrial ROS formation on isolated hippocampi mitochondria, mitochondrial suspension were incubated with 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (final concentration of 10 μM) in respiratory buffer including KCl (130 mM), $MgCl_2$ (5 mM), NaH_2PO_4 (20 mM), ADP (1.7 mM), β-NADPH (0.1 mM), $FeCl_3$ (0.1 mM), pH 7.4. Mitochondrial fluorescence and light scattering were analyzed for at least 12000 counts per sample in the flowcytometry using the BD Biosciences FACS CalibureTM flowcytometer. Samples were gated on the forward/side scatter to exclude cell debris and clumps. A flowcytometer with the Flomax software, equipped with a 488-nm argon ion laser was used and fluorescence signals were obtained using a 530-nm band pass filter (FL-1 channel) (Gao et al., 2009; Hosseini et al., 2014; Amini-Khoei et al., 2017).

ATP levels. Briefly, 0.5 ml aliquot of isolated mitochondria homogenate in TCA (6%) was mixed with 0.5 mL of KOH 0.05 M (on ice), then, 1 mL deionized water was added; after 2 min, 650 μL of KH_2PO_4 (0.05 M) was added and vortexed. After filtering, ATP level in each sample was measured using luciferase enzyme as described in our previous work (Eskandari et al., 2012). Bioluminescence intensity was measured using Sirius tube luminometer (Berthold Detection System, Germany).

Glutathione (GSH) levels. Glutathione levels were determined using 5, 5′-dithiobis-(2-nitrobenzoic acid) or DTNB as the indicator. Briefly, 0.1 mL of supernatant was added into $0.1\,mol.L^{-1}$ of phosphate buffer and 0.04% DTNB in a total volume of 3.0 mL (pH 7.4). The developed color was measured at 412 nm using a spectrophotometer (UV-1601 PC, Shimadzu, Japan).

GSH content was expressed as $\mu g\,mg^{-1}$ protein (Jayakumar et al., 2014).

Experimental design

Experimental design of the study consisting of injections (colitis vs. control), number of animals in each experimental group, and molecular/behavioral assessments which were performed in each group is summarized in Table 1.

Statistical analysis

The sample size was calculated by power calculations using G power software (ver.3.1.7, Franz Faul, Universitat Kiel, Germany). We set α error at 0.05 and power (1-β) at 0.8 and the required total sample size per group was calculated as 6–8 in behavioral tests and 3–4 in molecular studies. Comparison between the groups was analyzed using t-test using the Graph-pad prism software (version 6). $P < 0.05$ was considered statistically significant.

RESULTS

Assessment of colitis

In order to validate the DNBS-induced colitis, we have assessed the possible effect of DNBS on macroscopic and histopathological features and scores, MPO activity and pro-inflammatory cytokines expression levels.

Macroscopic evaluation

As shown in Fig. 1, the macroscopic scores were significantly higher in DNBS-treated mice when compared with the saline-treated mice ($t = 8.612$, $df = 12$, $P < 0.001$).

Microscopic evaluation

In microscopic examination, epithelial damage and inflammatory cell infiltration were detected in all DNBS-treated samples. Massive neutrophilic infiltration restricted to the mucosa as well as goblet cell and crypt loss was evident, suggesting a pattern of colonic damage. The histopathological scores were significantly higher in DNBS-treated mice when compared with the saline-treated mice ($t = 5.1$, $df = 14$, $P < 0.01$, Table 3, Fig. 2).

Colitis increased MPO activity in the colon tissue

MPO activity value in DNBS-treated group was significantly increased in comparison with the control group ($t = 16.63$, $df = 12$, $P < 0.001$, Fig. 3).

Colitis increased expression of inflammatory genes in the colon tissue

As shown in Fig. 4B, DNBS-induced colitis was associated with significant increase in the expression levels of the colonic pro-inflammatory cytokines including *Il-1β* ($t = 5.454$, $df = 6$, $P < 0.01$), *Il-6*
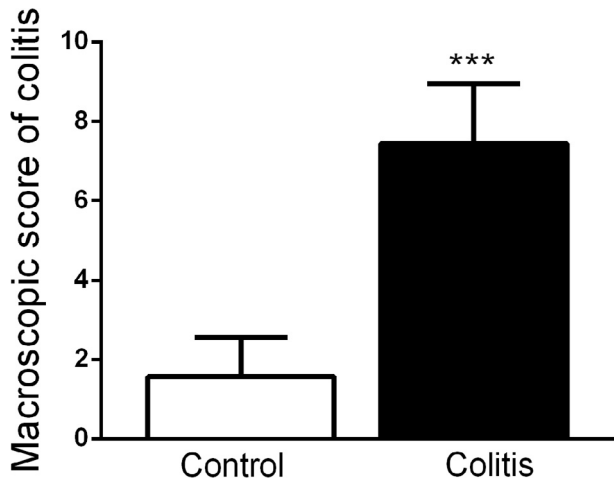
Fig. 1. Macroscopic scores of the colitis. Values are expressed as mean ± SD ($n = 8$) and were analyzed using t-test. ***$P < 0.001$ compared to the control group.

Table 3. Histopathologic scores of the colon samples. Histopathologic changes were scored semi-quantitatively. Values are expressed as median and min–max ($n = 8$) and were analyzed using t-test. **$P < 0.01$ compared to the control group

Group no	Treatment	Pathologic score Median (min–max)
1	DNBS	4 (2–5)**
2	Control	1 (0–2)

($t = 3.669$, $df = 6$, $P < 0.05$), Tnf-α ($t = 3.457$, $df = 6$, $P < 0.05$), $Hmgb1$ ($t = 3.233$, $df = 6$, $P < 0.05$), Tlr-2 ($t = 2.681$, $df = 6$, $P < 0.05$) and Tlr-4 ($t = 5.112$, $df = 6$, $P < 0.01$) as compared with the control saline-treated group.

Colitis provoked behaviors relevant to depression and anxiety

Analyses revealed that applying colitis using DNBS induced depressive- and anxiety-like behaviors in animals. We demonstrated the impact of colitis on
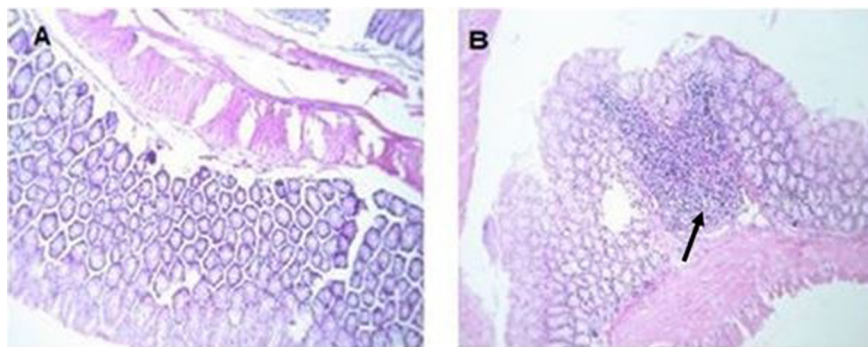

Fig. 2. Representative features of histopathologic evaluations provided from H & E-stained colon sections (×100). (A) The saline-treated group, the normal mucus layer and crypts without leukocyte infiltration; (B) The DNBS-treated group, mucosal layer with leukocyte infiltration (arrow).
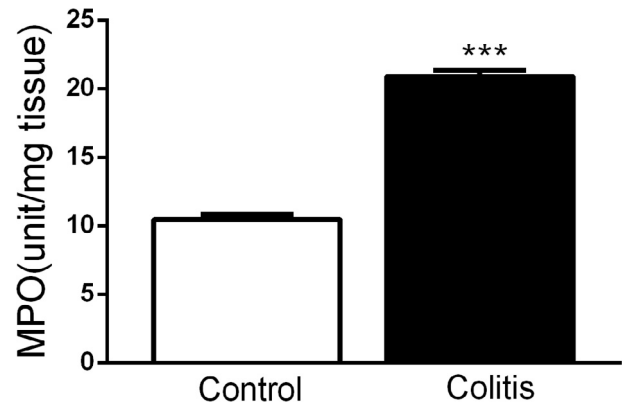

Fig. 3. Myeloperoxidase (MPO) activity in the colitis. Data are expressed as mean ± SEM ($n = 8$) and were analyzed using t-test. ***$P < 0.001$ compared to the control group.

depressive-like behaviors using FST, TST, splash test and SPT. The possible effect of colitis on locomotor activity and anxiety-like behaviors were determined using OFT, HBT, and EPM.

Colitis increased immobility time in the FST and TST

Results showed that the immobility time, main indicator of despair behavior, increased in DNBS-treated animals when compared with control groups in the FST ($t = 8.945$, $df = 14$, $P < 0.001$, Fig. 5A) and TST ($t = 3.465$, $df = 14$, $P < 0.01$, Fig. 5B).

Colitis decreased grooming activity time in the splash test

As shown in Fig. 5C, colitis caused self-care disturbance and significantly reduced grooming activity time in the splash test in comparison with the control group ($t = 7.443$, $df = 14$, $P < 0.001$).

Colitis decreased sucrose intake in the SPT. Our finding determined that colitis was accompanied with an anhedonic condition. SPT results showed that induction of colitis with DNBS significantly reduced sucrose consumption in comparison with control mice ($t = 5.99$, $df = 14$, $P < 0.001$, Fig. 5D).

Colitis decreased time spent in central zone without change in locomotor activity in the OFT

In the OFT, there is no significant difference in the total distance moved (horizontal activity) ($t = 0.3119$, $df = 14$, $P > 0.05$, Fig. 6A) and number of rearings (vertical activity) ($t = 0.6544$, $df = 14$, $P > 0.05$, Fig. 6B) between colitis and control groups. As shown in Fig. 6C, DNBS-treated animals spent less time in the central zone of the OF apparatus in comparison with the control animals ($t = 3.035$, $df = 10$, $P < 0.05$).
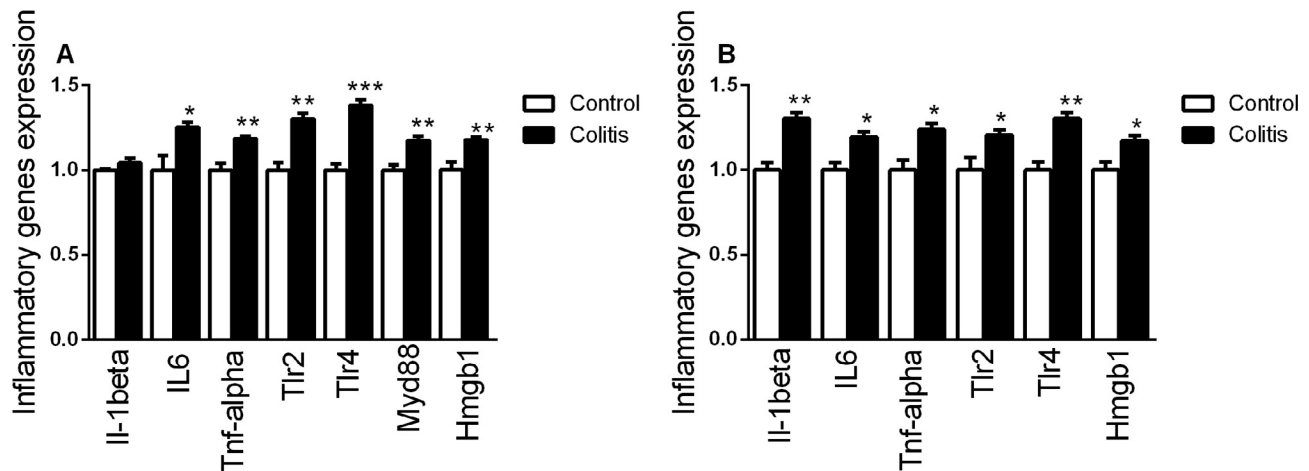
Fig. 4. The expression of Il-1β, Tnf-α, Tlr4, Tlr2, Il-6 and Hmgb1 in the hippocampus (A) and the colon (B) tissues were determined by qRT–PCR. Data are shown as mean ± SEM ($n = 4$) and were analyzed using t-test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control counterparts. Il-1β: interleukin-1 beta, Tnf-α: tumor necrosis factor alpha, Tlr4: toll-like receptor 4, Tlr2: toll-like receptor 2, Il-6: interleukin 6 and Hmgb1: high mobility group box 1protein.
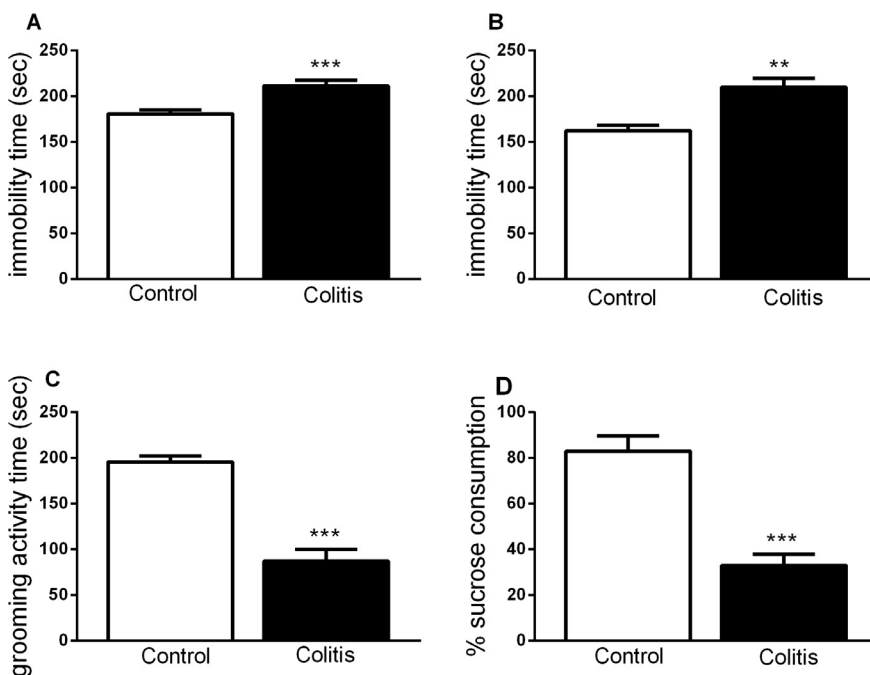

Fig. 5. Effects of colitis on depressive-like behaviors including immobility time in the FST (A), immobility time in the FST (B), grooming activity time in the splash test (C) and percent of sucrose consumption in SPT (D). Values are expressed as the mean ± S.E.M. ($n = 8$) and were analyzed using t-test. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group. FST: forced swimming test, SPT: sucrose preference test.

Colitis decreased number of head-dip in the HBT

As shown in Fig. 6D, colitis caused a significant decrease in number of head-dips in IBD group when compared with control animals ($t = 3.402$, $df = 14$, $P < 0.01$).

Open arm time and entries decreased in colitic mice in the EPM

In the EPM, percentage of spent time in the open arms and percentage of open arms entries were evaluated as variables relevant to anxiety-like behaviors. In comparison with the control animals, DNBS administration remarkably decreased percentage of spent time in the open arms ($t = 2.331$, $df = 14$, $P < 0.05$, Fig. 6E) as well as percentage of open arms entries ($t = 4.122$, $df = 14$, $P < 0.01$, Fig. 6F) in IBD mice.

Colitis increased expression of genes related to inflammation in the hippocampus

Fig. 4A shows the effects of IBD on expression of genes related to inflammatory markers. t-test analysis demonstrated the over-expression of Tlr-2 ($t = 5.421$, $df = 6$, $P < 0.01$), Tlr-4 ($t = 7.131$, $df = 6$, $P < 0.001$), Myd88 ($t = 4.330$, $df = 6$, $P < 0.01$) and Hmgb1 ($t = 3.745$, $df = 6$, $P < 0.01$) in hippocampus of IBD groups in comparison with the control animals. Also, significant rise in expression of Tnf-α ($t = 4.272$, $df = 6$, $P < 0.01$) and Il-6 ($t = 2.771$, $df = 6$, $P < 0.05$) was observed in the hippocampus of the colitic mice in comparison with the control mice. Our findings showed that there is no significant difference in the expression of Il-1β ($t = 5.1$, $df = 14$, $P > 0.05$) and nlrp3 ($t = 1.324$, $df = 6$, $P > 0.05$) in the hippocampus of colitic mice when compared with the control mice.

In addition, as presented in Fig. 7, colitis significantly decreased Bdnf expression in hippocampus in comparison with saline-treated mice ($t = 2.594$, $df = 6$, $P < 0.05$).
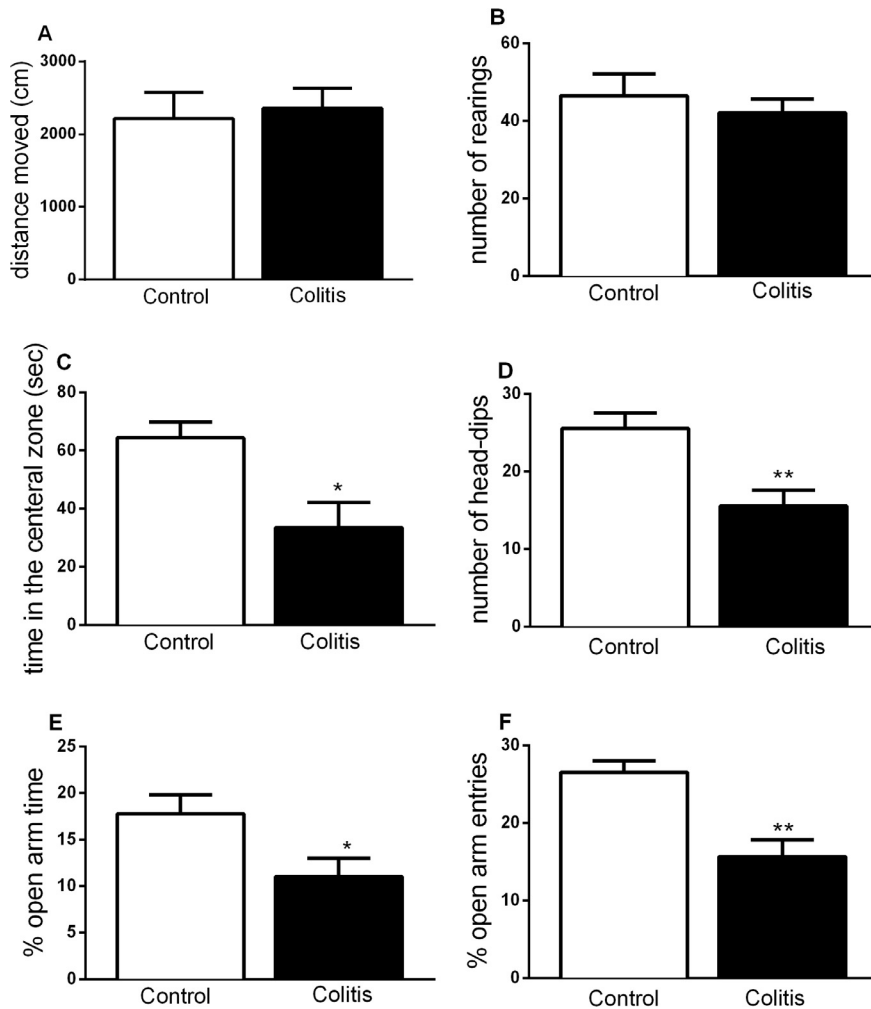
Fig. 6. Effect of colitis on behaviors related to anxiety and locomotor activity. Total distance moved in the OFT (A), number of rearings in the OFT (B), time spent in the central zone in the OFT (C), number of head-dips in the HBT (D),% open arm time in the EPM (E), and % open arm entries in EPM (F). Values are expressed as the mean ± S.E.M. ($n = 8$) and were analyzed using t-test. $^*P < 0.05$ and $^{**}P < 0.01$ compared to the control group. OFT: open-field test, EPM: elevated plus maze, HBT: hole-board test.
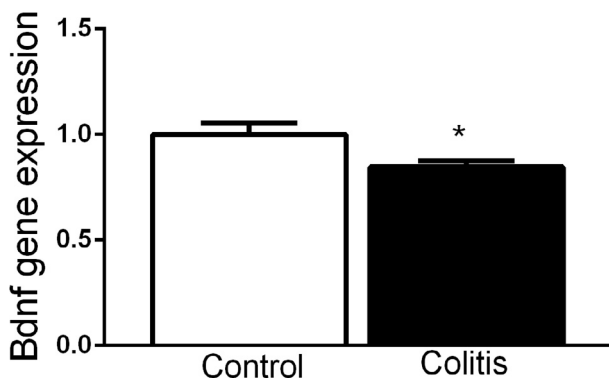

Fig. 7. The expression of Bdnf in the hippocampus was determined by qRT-PCR. Data are shown as mean ± SEM ($n = 4$) and were analyzed using t-test. $^*P < 0.05$ compared to the control counterparts. Bdnf: brain-derived neurotrophic factor.

Colitis induced mitochondrial dysfunction and NO overproduction in the hippocampus

The effects of colitis on hippocampal mitochondria parameters including GSH and ATP levels as well as ROS production were presented in Table 4. Results obtained from t-test analysis revealed that there was a significant decrease in GSH levels in the colitic group when compared with the control group ($t = 6.979$, $df = 8$, $P < 0.001$). Also, a significant decrease in ATP levels was observed in the colitic mice as compared with the controls ($t = 18.97$, $df = 8$, $P < 0.01$).

Furthermore, assessment of ROS formation was performed in 2 time intervals (5 min and 45 min) in the IBD and the control groups. Results obtained from t-test demonstrated that colitis significantly increased levels of ROS in the hippocampus in intervals 5 min ($t = 6.397$, $df = 11$, $P < 0.0$) and 45 min ($t = 4.545$, $df = 14$, $P < 0.001$) in comparison with control group (Table 5).

We have also evaluated the impact of colonic inflammation on hippocampal nitrite levels. Analysis revealed that colitic animals have a higher level of hippocampal nitrite content in comparison with the controls ($t = 8.497$, $df = 8$, $P < 0.001$, Table 4).

DISCUSSION

Results of the current study showed that depressive- and anxiety-like behaviors in an animal model of CD are associated with increased transcription of the genes relevant to innate immunity in the hippocampus. In addition, altered expression of these genes was accompanied by an overproduction of ROS and NO in hippocampus of animals. We found that colonic inflammatory responses were associated with the infiltration of inflammatory cells and the increased expression of the genes involved in TLRs pathway in intestinal tissue. These alterations were accompanied by increased transcriptional levels of the genes relevant to innate-immunity in the hippocampus of colitic mice. Further, activation of innate immunity was associated with behavioral abnormalities and oxidative stress in the hippocampus. These results suggest that association between peripheral and central immune-inflammatory responses play a critical role in the co-occurrence of depression and anxiety in CD.

In the current work, using an experimental model of colitis, we observed a significant inflammation in the

Table 4. Effect of colitis on GSH, ATP, and nitrite levels in the hippocampus: Values are expressed as Mean ± S.D. ($n = 4$) and were analyzed using t-test. [**]$P < 0.01$ and [***]$P < 0.001$ compared with control group. GSH: glutathione, ATP: adenosine triphosphate

	GSH (μg/mg protein)	ATP (nmol/mg tissue)	Nitrite (nmol/mg protein)
Control	14.7 ± 2.3	3.2 ± 0.2	79.48 ± 2.8
Colitis	5.36 ± 1.7[***]	1.28 ± 0.13[**]	147.4 ± 3.2[***]

Table 5. % increase of ROS formation in hippocampus after induction of colitis. Values are expressed as Mean ± S.D. ($n = 4$) and were analyzed using t-test. [**]$P < 0.01$ and [***]$P < 0.001$ compared with control group. ROS: reactive oxygen species

Groups	DCF fluorescence intensity (%)	
	5 min	45 min
Control	3 ± 2	9 ± 1
Colitis	12 ± 1[**]	157 ± 14[***]

colonic tissue that was confirmed by pathological and molecular evaluations. Infiltration of inflammatory cells such as PMN cells and macrophages into colonic tissue was accompanied by an increase in the MPO level and upregulation of the *Tlr-2* and *4* and *Hmgb1* as well as pro-inflammatory cytokines including *Tnf-α, Il-1β,* and *Il-6*. Ample evidence has demonstrated the significant contribution of pro-inflammatory cytokines in the pathogenesis of IBD (Strober and Fuss, 2011; Senhaji et al., 2016). Inflamed intestinal barrier contains antigen-presenting cells such as macrophages which produce several inflammatory factors (Papadakis and Targan, 2000). In this regard, IL-1β is known to increase the para-cellular permeability of the intestinal mucosa/epithelium, and has been suggested as a determinant factor for the initiation of disease (Al-Sadi et al., 2008; Neurath, 2014). Also, TNF-α and IL-6 are known to mediate the majority of systemic inflammatory responses in IBD, which account for the disease progression, perpetuation and comorbid difficulties (Leppkes et al., 2014; Neurath, 2014; Waldner and Neurath, 2014). These results are in line with previous investigations, which have reported that experimental models of colitis have severe colonic damage and pervasive inflammatory responses in the intestinal tissue (Martin-Subero et al., 2015; Heydarpour et al., 2016).

We used validated and qualified behavioral tests for the evaluation of anxiety- and depressive-like behaviors in rodents. We found that the colitic mice exhibited behaviors relevant to anxiety and depression 3 d following DNBS-induced injury. Increased immobility time in both FST and TST reflects the inability of rodents to cope with an acute unescapable challenge reflecting the depressive-like behaviors similar to behavioral despair observed in depressed people (Cryan and Holmes, 2005). Results of this work revealed that the colitic mice not only had a significant increase in the immobility time, but also had a decline in sucrose preference and consumption as well as self-care disturbance

in SPT and splash test. A decrease in consumption of sucrose 1% in the SPT and low response (decrease in grooming activity time) to sucrose 10% in splash test have been reported as behaviors associated with anhedonia and motivational difficulties in rodents (Der-Avakian and Markou, 2012; Marrocco et al., 2014; Petit et al., 2014; Amiri et al., 2015b). Also, the depressive behaviors of colitic animals in our experiments were not related to the changes of locomotion. Although the FST and TST are trusted behavioral tests, false negative results might be obtained with sedation and sickness conditions. However, the results we observed in this study do not seem to be associated with locomotion changes, since DNBS-induced colitis did not change the locomotion in the OFT. Further, we have shown that colitis provoked anxiety-like behaviors in OFT (decrease in spent time in the central zone), EPM (decrease in both frequency and spent time in open arms), and HBT (decrease in the number of head-dips). This behavioral profile indicates that the induction of colitis in mice is associated with the co-occurrence of anxiety- and depressive-like behaviors. Clinical and preclinical investigations have recently demonstrated that not only intestinal damage in IBD induces behaviors relevant to affective disorders, but also inflammatory factors play a part in mediation of these negative behavioral changes (Hassan et al., 2014; Pellissier et al., 2014; Heydarpour et al., 2016). Prevalence of depression and/or anxiety is more common in patients with IBD in comparison with the general population, suggesting that patients with IBD require psychiatric care (Magni et al., 1991; Addolorato et al., 1997; Kurina et al., 2001).

There is a consensus agreement that oxidative challenge and inflammatory pathways are bidirectional bridges that link diversity of systemic diseases to negative affective disorders (Slavich and Irwin, 2014; Martin-Subero et al., 2015). Emerging lines of research have highlighted the role of brain–gut axis in the comorbidity of psychiatric disorders and bowel diseases (Kennedy et al., 2014; Severance et al., 2014; Mayer et al., 2014a, 2014b). It has been well documented that immune-inflammatory cascades play a pivotal role in the development of negative affective disorders such as anxiety and depression (Irwin and Miller, 2007; Maes, 2011). Recent investigations have demonstrated that activation of TLRs in cortico-limbic regions contribute to pathobiology of several brain disorders including anxiety and depression (Dantzer et al., 2008; Wohleb, 2013). In the current study, depressive- and anxiety-like behaviors in colitic mice were accompanied by an increase in the expression of genes relevant to innate immunity such as TLRs and NLRP3 pathways. Overexpression of the *Tlr2* and *Tlr4* in the hippocampus was associated with upregulation of *Myd88* as the main regulatory protein of the TLRs pathway. Stimulation of TLR-4 and TLR-2 activate several signaling pathways in the CNS which lead to the initiation of inflammatory responses. Recently published results by our colleagues showed that TNBS-induced colitis induces behavioral despair in animals and these behavioral changes are associated with a significant increase in hippocampal TNF-α and iNOS protein levels

(Heydarpour et al., 2016). Moreover, inhibition of iNOS by aminoguanidine reversed the despair behavior of animals in the FST and decreased both iNOS and TNF-α level in the hippocampus (Bakunina et al., 2015; Heydarpour et al., 2016). In this work, we observed that DNBS-induced colitis not only increased nitrite contents in the hippocampus, but also induced mitochondrial abnormalities and oxidative challenge. We found a considerable decrease in hippocampal ATP levels, suggesting that mitochondria were not able to produce enough energy supply following DNBS challenge. Using flowcytometry, a valid and quantitative tool for the measurement of mitochondrial ROS, we observed a time-dependent increase in mitochondrial derived ROS suggesting the abnormal function of mitochondria in the hippocampus. Also, a significant decrease in the GSH levels, as the main antioxidant in the brain, indicates that overproduction of ROS and NO causes oxidative challenge in the hippocampus. In this regard, previous investigations have highlighted the role of oxidative challenge and nitrergic system in modulation of anxiety- and depressive-like behaviors (Amiri et al., 2015a; Haj-Mirzaian et al., 2016). Mitochondria are intracellular organelles which have pivotal role in ATP production in the cells (Calabrese et al., 2001). Mitochondrial dysfunction as defect in biochemical cascade or damage to the mitochondrial respiratory chain has been recommended as a main factor in the pathogenesis of neuropsychiatric disorders such as anxiety and depression (Rezin et al., 2009; Hovatta et al., 2010; Tobe, 2013). Suggesting, brain metabolism impairment and decrease in mitochondrial ATP can be considered as a mechanism underlying psychiatry disorders (Fattal et al., 2006; Burroughs and French, 2007; Rossignol and Frye, 2015). In this regard, it is demonstrated that potent anxiolytic and antidepressant agents may improve and protect mitochondrial efficiency and function indicating mitochondrial function may be linked to the pathophysiology and treatment of behavioral/mood disorders (Einat et al., 2005; Burroughs and French, 2007).

Clinical and preclinical studies have suggested that peripheral inflammatory responses in IBD trigger the initiation of immune-inflammatory pathways in the brain through different pathways (Maes et al., 2011b; Martin-Subero et al., 2015). Despite the extensive research in the bidirectional effects of brain–gut axis, it is not clear how inflammatory factors in the bowel can influence behavioral profile sufferings. Focusing on animal studies, recent studies have demonstrated that TNBS-induced colitis is able to enhance neuronal excitability and inflammatory responses in the hippocampus through increasing the activity of glutamatergic system and TNF-α (Neurath et al., 2000; Boissé et al., 2003; Riazi et al., 2008). Although several mechanisms have been proposed to mediate comorbidity of behavioral difficulties in IBD, most of researchers suggest that cytokines are key mediators of such responses (Mackner et al., 2011). Our results revealed that upregulation of *Tlr2* and *Tlr4* genes in the hippocampus was associated with an increase in the expression of inflammatory genes (MyD88, IL-6, and TNF-α) as well as oxidative and nitrosative stress. However, the main question is that how TLR-2 and TLR-4 were activated in the hippocampus of mice which experienced DNBS-induced colitis. To answer this question, we focused on the expression of the Hmgb1 gene.

It has been well documented that HMGB1 modulates the systemic inflammatory responses in vast majority of disorders and in case of IBD, this factor has been suggested as the clinical marker for IBD diagnosis in the stool of patients (Alex et al., 2007; El Gazzar, 2007; Chassaing et al., 2012). In addition, HMGB1 is known as a well-known DAMP that accounts for activation of both TLR-2 and TLR-4 in variety of inflammatory diseases (Yu et al., 2006; Mudaliar et al., 2013). In line with previous reports, we observed an upregulation in the HMGB1 gene not only in the colon tissue, but also in the hippocampus of colitic mice indicating that this molecule at least in part, mediates the primary inflammatory responses in the CNS which results in appearance of behavioral comorbidities. Hippocampus has high density of microglial cells. It has been suggested that activation of hippocampal microglial implicated in the pathophysiology of psychiatric and stress-related disorders (Rohan Walker et al., 2013; Brites and Fernandes, 2015). In this context, it has been shown that activation of HMGB1 is associated with depression while blockage of HMGB1 attenuated the depressive-like behaviors (Weber et al., 2015; Wu et al., 2015). Further, we also found that the expression of brain-derived neurotrophic factor (BDNF) was significantly lower in colitic animals. This neurotrophic factor has a pivotal role in neuronal survival, regulation of neuronal differentiation, migration and activity-dependent synaptic plasticity (Duman et al., 2000; Vutskits et al., 2001). It has been demonstrated that decreased BDNF expression in the hippocampus is associated with pathophysiology of depression-like behaviors. The fundamental role of BDNF in pathophysiology of neuropsychiatric diseases is approved by this fact that its level can be improved by neuropsychiatric medications, such as antidepressants, mood stabilisers and antipsychotics (Harrisberger et al., 2015).

It should be noted that we report DNBS-induced alterations at transcriptional level, and our results would be improved if we measure these alterations at protein levels. In addition, we could improve results of this study by applying a pharmacological treatment to animals (such as an anti-inflammatory compound or antioxidant) to show the involvement of oxidative stress and inflammatory responses in behavioral abnormalities following colitis. However, our results showed that colitic mice exhibit a wide range of behaviors relevant to anxiety and depression. Our results also revealed that altered hippocampal energy and redox status may play a role in aggravating the inflammatory signaling following induction of colitis in mice. Although we showed the increased transcription of *Hmgb1* in both central and peripheral tissues, we could not show that HMGB1 triggers the inflammatory responses in the hippocampal formation. We believe that further research is needed to investigate the role of toll-like receptor pathway and mitochondrial function in the co-occurrence of anxiety and depression in IBD.

Interestingly, there are a variety of compounds that have been considered as useful for treating both IBD and mood disorders. Plant compounds extracted from Glycyrrhiza uralensis (Chinese licorice), Magnolia officinalis, Zingiber officinale (ginger), Salvia miltiorrhiza and curcumin exert anti-inflammatory effects through interacting with TLR-4 pathway (Lucas and Maes, 2013). For instance, epigallocatechin-3-gallate (EGCG) is a polyphenol compound found in green tea, and is able to inhibit the MyD88 signaling (Youn et al., 2006). Further, 6-shoagol, as an active compound of ginger, is able to block the activity of inhibitor-κB kinase (Park et al., 2009). Many studies have shown that most of these herbs are useful for the treatment of IBD and depression (Xu et al., 2005; Zhao et al., 2008; Sun et al., 2009; Ung et al., 2010). Focusing on mitochondrial function and oxidative & nitrosative stress, studies have shown that reducing mitochondrion-induced oxidative stress have modulating effects on TLR pathway and is useful to alleviate symptoms in both depression and IBD (Tirosh et al., 2007; Lucas and Maes, 2013; Heydarpour et al., 2016; Sonei et al., 2017). For example, N-acetylcysteine (NAC) is a strong radical scavenger that showed therapeutic effects in both depression and IBD (Cetinkaya et al., 2005; Magalhães et al., 2011). In addition, inhalation or consumption of hydrogen (hydrogen-enriched water) is able to decrease the activity of TLR4 pathway by inhibiting NF-kB activity (Ito et al., 2011; Ohno et al., 2012). It has been shown that consumption of hydrogen-enriched water is able to alleviate the mitochondrial dysfunction and inflammatory responses (Li et al., 2012; Xie et al., 2012). Interestingly, studies have shown the efficacy of hydrogen consumption in the treatment of both colitis and psychiatric disorders (Carbonero et al., 2012; Ghanizadeh and Berk, 2013).

CONCLUSION

Using an experimental animal model of Crohn's disease, our results showed that behavioral abnormalities in early stages of disease are associated with immune-inflammatory responses (at transcript level) in both colon and hippocampus. We demonstrated that DNBS-induced colitis is able to provoke anxiety- and depressive-like behaviors in animals. These behavioral changes were accompanied by altered energy metabolism and oxidative and nitrosative stress in the hippocampus as well as increased expression of genes in TLR-pathway. In addition, we suggest that HMGB1, as an endogenous ligand for Tlr-2 and Tlr-4, may play a role in the activation of inflammatory responses in the brain and occurrence of abnormal behaviors in animals.

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