Iranian Journal of Basic Medical Sciences
ijbms.mums.ac.ir

Trigonelline protects against alcohol-induced brain damage by inhibition of oxidative stress, TLR4/NF-κB/proinflammatory cytokines pathway, and apoptosis

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ARTICLE INFO

Article type:
Original

Article history:
Received: Jan 25, 2026
Accepted: May 9, 2026

Keywords:
Alcohol
Neuroinflammation
Neurotoxicity
TLR4/NF-κB
Trigonelline

ABSTRACT

Objective(s): Brain injury is one of the most predominant complications following excessive alcohol consumption. Oxidative, inflammatory, and apoptotic processes are the essential mechanisms involved in alcohol-induced brain damage. Trigonelline is a natural compound that has a variety of pharmacologic activities. The present study investigated the protective effect of trigonelline in alcohol-induced brain injury and its underlying mechanisms.

Materials and Methods: Adult male mice (C57BL/6) were exposed to binge ethanol (6 g/kg/day, by gavage) and treated with trigonelline (50 and 100 mg/kg/day, orally) for 6 days. Mice were sacrificed and the brain tissues were dissected for experimental assessments.

Results: The results showed that trigonelline alleviated alcohol-induced locomotor impairment and brain oxidative damage by decreasing lipid peroxidation and protein oxidation. Trigonelline restored the levels of protective antioxidants (GSH, SOD, and HO-1) and reduced the levels of ICAM-1 and MPO in the brains of mice exposed to alcohol. Trigonelline significantly reduced alcohol-induced brain inflammation by the inhibition of iNOS/NO, TLR4, NF-κB, and proinflammatory cytokines (TNF-α, IL-6, IL-1β, and TGF-β1). Moreover, trigonelline treatment reduced the levels of caspase-3, cytochrome c, and TUNEL positive cells in the brains of alcohol-exposed mice.

Conclusion: These findings suggest that trigonelline protects brain against alcohol intoxication by inhibition of oxidative and inflammatory and apoptotic responses. Therefore, trigonelline may serve as a potential therapeutic approach for the protection of brain damage associated with binge alcohol consumption.

▶ Please cite this article as:
Amirshahrokhi K, Niapour A, Imani M. Trigonelline protects against alcohol-induced brain damage by inhibition of oxidative stress, TLR4/NF-κB/proinflammatory cytokines pathway, and apoptosis. Iran J Basic Med Sci 2026; 29: 1079-1088. doi: https://dx.doi.org/10.22038/ijbms.2026.94209.20306

Introduction

Alcohol (ethanol) is one of the most commonly abused and addictive substances in many countries. Consuming alcohol in high amounts is dangerous for human health and may lead to death. Alcohol intoxication by binge drinking can cause serious and permanent dysfunction in multiple organs, especially in the CNS. Ethanol readily crosses the blood-brain barrier and induces neurotoxicity, leading to impairment of brain functions, including cognition and motor activity. Oxidative stress, neuronal inflammation, and apoptosis are the mechanisms suggested to be involved in the pathogenesis of alcohol-associated brain injury (1-3).

Alcohol is metabolized by alcohol dehydrogenase in liver and other organs, including the brain and produces acetaldehyde, which is then oxidized into acetate. These metabolizing processes generate reactive oxygen species (ROS) and disturb the antioxidative defense mechanisms of the cells. Overproduction of ROS in the brain is responsible for neurotoxicity by oxidative changes of biomolecules such as proteins, phospholipids, and nucleic acids (4-6). It has also been reported that alcohol-induced free radicals can impair mitochondrial function and increase the levels of pro-apoptotic proteins such as cytochrome c and caspase-3, which may lead to the neurodegeneration process in the brain (7).

Alcohol can profoundly induce brain inflammation through activation of inflammatory transcription factors and increase of pro-inflammatory cytokines (8). Alcohol-induced neuroinflammation is associated with activation of Toll-like receptors (TLRs), which play an essential role in innate immunity and inflammatory cascades (9). Exposure to alcohol stimulates NF-κB and may increase the levels of inflammatory mediators such as phospholipase A2, cyclooxygenase, and iNOS in the brain cells. Activation of NF-κB promotes the production of several inflammatory cytokines (TNF-α, IL-1β, IL-6, and MCP-1) which may result in significant neuroinflammation (10, 11). All of these events ultimately lead to brain damage and consequently neurological and psychological symptoms of alcoholism.

Various studies have shown that natural products and dietary supplements could be used to prevent

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or treat alcohol-induced disorders (12). Trigonelline (N-methylnicotinic acid) is a methylated derivative of niacin (vitamin B3) that naturally occurs in several plant species, such as fenugreek (Trigonella foenum-graecum) seeds and coffee beans. Trigonelline, as one of the main active ingredient of herbal drugs, is commonly used for the treatment of several human disorders and is safe for clinical applications (13). It exerts significant pharmacological properties, including anti-inflammatory, anti-apoptotic, antioxidant, and neuroprotective properties. A variety of studies have reported that trigonelline can be effective in the treatment of diabetes mellitus (14), allergic asthma (15), age-related muscle loss (16), alcohol-induced heart damage (17), Alzheimer's disease (18), seizure (19), LPS-induced memory impairment (20), and cerebral ischemia/reperfusion injury (21, 22).

Based on the background mentioned, we proposed that trigonelline could inhibit oxidative stress, inflammation and apoptosis induced by high-dose ethanol in the brain. In the present study, we carried out a mouse model of binge alcohol exposure to study the potential neuroprotective effect of trigonelline against alcohol-induced brain damage and the underlying mechanisms.

Materials and Methods

Animals

Male C57BL/6 mice (7 weeks old) (20–22 g) (Pasteur Institute, Tehran, Iran) were utilized in the experiments. All mice were housed in plexiglass cages and kept in a room with controlled conditions (temperature of 22 °C ± 2 °C and 12-hr light/dark cycle). Animals received laboratory food and water *ad libitum*. Animal methods were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. All experiments of this work were also permitted by the Research Ethics Committee of Ardabil University of Medical Sciences, Ardabil, Iran (IR.ARUMS.AEC.1403.022).

Experiment design

Mice were randomly allocated to four groups (n = 6). Mice in the normal control group received water. Mice in the alcohol group received ethanol (6 g/kg, oral) by gavage once daily for 6 days. Animals in the two treatment groups received ethanol (6 g/kg. oral) and trigonelline 50 mg/kg or 100 mg/kg by gavage once daily for 6 days. The doses of alcohol and trigonelline were determined according to the previous studies (23, 24). Alcohol at the doses of 5–6 g/kg is a range often used in mouse models for binge drinking or acute exposure to ethanol. In our study we used trigonelline at the doses of 50 and 100 mg/kg/day (orally), which correspond to the doses of 4–8 mg/kg/day in human. Moderate to heavy coffee drinkers can be exposed to these concentrations of trigonelline. Trigonelline (TR951010, Mashhad, Iran) was dissolved in water and used one hour after each dose of alcohol. All mice were anaesthetized and sacrificed at the end of the experiment, and the brain tissues were dissected and then washed with cold normal saline. The whole brain tissue was divided into two hemispheres immediately. One hemisphere was fixed overnight in 4% formaldehyde (for 24 hr) for immunofluorescence evaluation and the other hemisphere was frozen in liquid nitrogen for biochemical and RNA analysis.

To prepare tissue homogenate, collected brain samples were homogenized in phosphate buffered saline (10 mM, pH 7.4). The tissue concentration of each sample was 200 mg/ml. The homogenized solutions were centrifuged (11,000 g, 10 min, 4 °C) and the supernatants were separated and stored in a freezer at -70 °C until assay.

Behavioral analyses

The behavioral assessment was performed at the last day of the experiment. The open field test was used to evaluate locomotor activity and physical function of mice. Test arena used in our study was made up of plexiglass (40 cm length × 30 cm width × 20 cm height) which was subdivided into equal squares. In each test, a mouse was placed in the arena and allowed to freely move for 5 min. The number of lines crossed by each animal was recorded over a 5 min period. The average number of line crossing in 1 minute was calculated and reported as the locomotion activity of each animal.

Assessment of oxidant and antioxidant markers

The levels of malondialdehyde (MDA) as the end-product of lipid peroxidation in all brain tissue samples were determined by using thiobarbituric acid (TBA) reagent. One hundred microliter of the supernatant was added to 200 µl of a solution containing TBA (0.1 M) and trichloroacetic acid (TCA 20% w/v). The mixture was vortexed and heated in 90 °C for 40 min. After cooling and centrifugation the absorbance of the colored product was measured by a microplate reader (BioTek, USA) at 532 nm.

Carbonyl protein as a biomarker of oxidized proteins was measured using 2,4-dinitrophenylhydrazine (DNPH) substance. Fifty microliter of each sample supernatant was mixed with 100 µl of DNPH (0.1 M) dissolved in HCl (2 N) in a 1.5-ml Eppendorf tube. After incubation at room temperature for one hour, 100 µl of TCA (20%) was added to the sample tubes and centrifuged (10,000 g for 5 min). The resultant pellets were washed using a mixture of ethanol and ethyl acetate (1:1 v/v). Next, each pellet was dissolved in 150 µl of guanidine hydrochloride (6 M) and the samples were transferred to a 96-well plate. The absorbance was detected at a wavelength of 370 nm and the level of protein carbonyl content was calculated by the molar absorbance coefficient of 22,000 M^{-1} cm^{-1}. The results were stated as nmol protein carbonyl/mg tissue.

The contents of reduced glutathione (GSH) as an endogenous antioxidant were analyzed using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) reagent. In an Eppendorf tube, 50 µl of each sample supernatant was added to 50 µl of TCA (10% w/v) solution and centrifuged. The resultant clear samples were separated and equal volumes of the samples transferred to a 96-well plate. After that, 150 µl of DTNB (1 mM), dissolved in sodium phosphate buffer (pH 7.8), was added to each well. The absorbance of produced yellow color was measured by a microplate reader at 412 nm.

The activity of superoxide dismutase and heme oxygenase-1 enzymes in tissue supernatants were detected using ELISA kits (SOD, Cayman Co, 706002, USA; HO-1, 1026-M9648, Padginteb Co. Tehran, Iran) following the manufacturer's manual.

Evaluation of MPO, ICAM-1 and NO

The activity of myeloperoxidase (MPO) in the tissue samples was assessed according to the method of hydrogen peroxide (H_2O_2)-dependent colorimetric oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) (25). Briefly, 50 µl of sample was combined with 50 µl of TMB solution (in DMSO). The reaction was started by adding 50 µl of potassium phosphate buffer (50 mM, pH 5.4) containing H_2O_2 (25 mM). MPO activity was detected by monitoring the changes in absorbance at 370 nm during 1 min using a

microplate reader. The concentration of NO and ICAM-1 in the brain tissue supernatants were measured using mouse-specific ELISA kits (NO, Cayman Co, 730001, USA; ICAM-1, ZB-10039C-M9648, ZellBio Gmbh, Ulm, Germany) and the results were stated as micromole per mg tissue.

Real time RT-PCR

The total RNA from each sample was extracted with Trizol reagent and reverse-transcribed to cDNA by a commercial cDNA synthesis kit. Quantitative real-time PCR amplification was done with SYBR Green master mix and synthetic primers specific to each gene by an ABI StepOne RT-PCR instrument (USA). Expression levels of mRNA relative to control were calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as a housekeeping gene for normalization of the expression level of each target gene. The primer sequences were described in our previous studies (26, 27).

Immunofluorescence analysis

The brain samples were assessed by immunofluorescence staining for TLR4 and p-NF-κB p65 expression. Briefly, the tissue sections were deparaffinized with xylene and rehydrated in graded ethanol. Antigens were retrieved by incubation with citrate buffer (pH 6.2) for 20 min. After washing by tris-buffered saline (TBS) and Triton X-100, slides were blocked in bovine serum albumin (BSA 1%) in TBS for 2 hr at 20 °C. After washing, the slides were incubated with primary antibodies (TLR4, sc-293072, and p-NF-κB p65, sc-136548, Santa Cruz, USA) overnight at 4 °C. Sections were incubated with fluorescent-conjugated secondary antibody at 20 °C for 1 hr. After washing, the sections were stained with DAPI (4′,6-diamidino-2-phenylindole), a blue fluorescent dye, to visualize cell nuclei. Slides were washed three times and dehydrated with graded ethanol and xylene. At the end, the slides were mounted with a coverslip and immunofluorescence images were taken by a fluorescence microscope (Olympus BX50). The mean fluorescence intensity was measured using ImageJ/Fiji software (version 1.8.0).

Assessment of inflammatory cytokines and apoptotic mediators

The levels of cytokines and apoptotic markers caspase-3 and cytochrome c in the tissue supernatants were determined using ELISA kits (TNF-α, ZB-10117C-M9648; IL-6, ZB-OEM21211103-121; IL-1β, ZB-OEM26211103-101; caspases-3, ZB-10445C-M9648; Cyt-C, ZB-10499C-M9648, ZellBio Gmbh, Ulm, Germany) according to the manufacturer's guidelines.

The number of apoptotic cells was quantified using the method of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The brain samples were fixed at room temperature with 4% paraformaldehyde. The tissue blocks were dehydrated in ethanol, embedded in OCT compound and cryosectioned into 10 μm thickness using a microtome. The sections were mounted on glass slides and processed for the TUNEL assay using an apoptosis detection kit (Millipore, USA). The tissue sections were stained with TUNEL and DAPI florescence dyes and observed under a fluorescence microscope. The percentage of TUNEL-positive cells, indicative of DNA fragmented cells, was measured by dividing the number of TUNEL-positive nuclei (green) by the total number of DAPI-stained nuclei (blue) in the same field (28).

Statistical analysis

The Shapiro-Wilk test was used to evaluate the normality of the data, and Levene's test was used to assess variance homogeneity. Statistically significant differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-test. The data were expressed as mean ± standard deviation (SD). A *P*-value less than 0.05 was accepted as statistically significant. SigmaPlot 14.0 software was used for creating graphs and statistical analysis.

Results

Trigonelline alleviates body weight loss and improves locomotion activity in mice exposed to high-dose alcohol

As displayed in Figure 1A, the body weight of mice exposed to high-dose alcohol was decreased during the experiment as compared to the control group. However trigonelline treatment (50 and 100 mg/kg) attenuated the body weight loss in mice exposed to alcohol. Behavioral analysis indicated that administration of high-dose alcohol (6 g/kg/day) significantly ($P<0.001$) impaired the locomotion activity of mice when compared to the normal mice (Figure 1B). Treatment with trigonelline significantly ($P<0.001$) improved the locomotion activity of mice exposed to alcohol. This result indicates that trigonelline alleviates alcohol-induced brain injury and then improves physical function of mice exposed to alcohol.

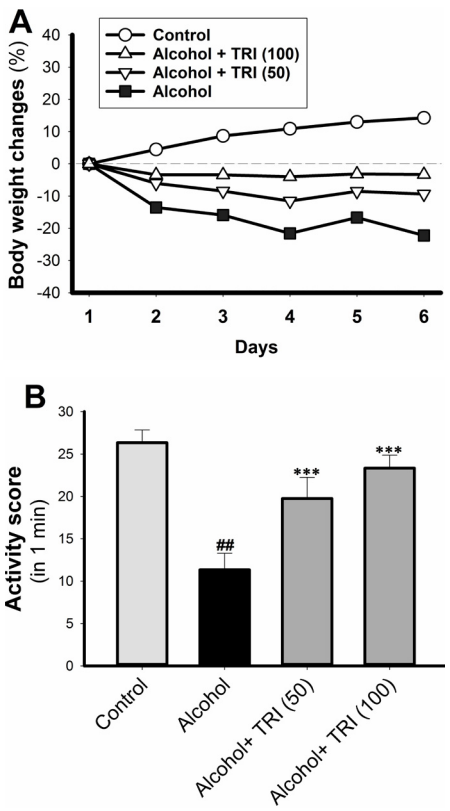

Figure 1. Effect of trigonelline on the body weight changes (A) and locomotor activity (B) in mice

The body weight change in each day compared to the weight of mice at the first day was measured. The change in body weight of mice in each group was expressed as percentage. Treatment with trigonelline (50 and 100 mg/kg/day) alleviated the body weight loss and improved locomotor activity of mice exposed to high-dose alcohol (6 g/kg). Data are presented as mean ± SD (n = 6). ##$P<0.001$ (compared with control group), ***$P<0.001$ (compared with alcohol group)

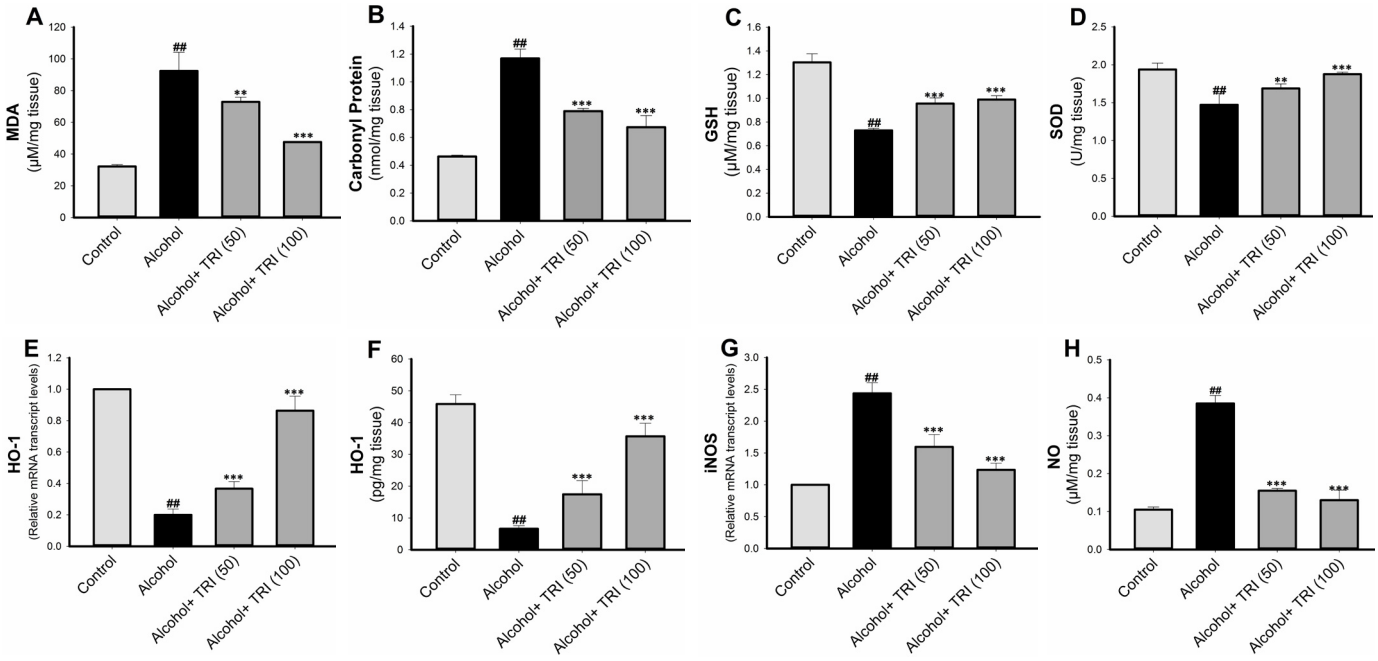

Figure 2. Effect of trigonelline on the brain levels of oxidative and anti-oxidative markers. Administration of trigonelline (50 and 100 mg/kg) in alcohol-exposed mice reduced the levels of MDA (A) and carbonyl proteins (B), and increased the levels of GSH (C), SOD (D), and HO-1 (E, F) in the brain tissue. Trigonelline treatment inhibited iNOS expression (G) and NO production (H) in the brain of mice exposed to alcohol
Data are presented as mean±SD (n=5). ##P<0.001 (compared with control group), **P<0.01, ***P<0.001 (compared with alcohol group)

Trigonelline inhibits alcohol-induced oxidative stress

To evaluate the ability of trigonelline to inhibit oxidative stress induced by ethanol, we measured the markers of oxidative damage and the levels of ROS-scavenging molecules in the brain tissue. As shown in Figures 2A and B, the levels of MDA as a lipid peroxidation product and protein carbonyl as an indicator of oxidized proteins were significantly increased in the animals exposed to alcohol as compared to the control mice (P<0.001). Administration of trigonelline (50 and 100 mg/kg) significantly reduced alcohol-induced MDA and carbonyl protein levels in the brain (P<0.001). Moreover, exposure to the high-dose alcohol (6 g/kg) depleted the levels of essential anti-oxidative factors GSH and SOD in the brain tissue as compared with those in the normal control mice (Figures 2C and D). In contrast, treatment with trigonelline significantly improved the levels of GSH and SOD in the brain of mice intoxicated by alcohol (P<0.001).

We assessed the level of HO-1 as an anti-oxidant biomarker. Administration of high-dose alcohol resulted in a low level of HO-1 expression and activity (P<0.001) in the brain tissue, while treatment with trigonelline significantly improved the levels of HO-1 (Figures 2E and F).

To determine whether excess levels of NO contribute to ethanol-induced brain injury, we assessed iNOS/NO signaling in the brain. Our results revealed that ethanol exposure up-regulated iNOS expression and dramatically increased NO production in the brain of mice as compared with those in the normal control animals (P<0.001) (Figures 2G and H). Administration of trigonelline (50 and 100 mg/kg) in mice exposed to alcohol significantly decreased iNOS expression and overproduction of NO in the brain tissue as compared to the alcohol group (P<0.001).

Trigonelline inhibits alcohol-induced TLR4/NF-κB activation and proinflammatory mediators

We assessed the levels of MPO and ICAM-1 as proinflammatory mediators. Binge alcohol exposure remarkably increased MPO activity and ICAM-1 level in the brain of mice as compared with the control group (P<0.001) (Figures 3A and B). However, trigonelline at both doses significantly decreased the levels of MPO and ICAM-1 in the treatment groups (P<0.05).

Immunofluorescence results showed that exposure to high-dose alcohol significantly increased the immunoreactivity of TLR4 in the brain tissue as compared to the control mice (P<0.001) (Figures 3C and D). Treatment with trigonelline at doses of 50 and 100 mg/kg reduced (P<0.001) expression of TLR4 in the brain of mice exposed to alcohol.

NF-κB as the master regulator of inflammation and apoptosis was assessed to investigate whether the inhibition of NF-κB exerts protective effects against alcohol-induced brain damage. Gene expression results revealed that the mRNA levels of NF-κB were higher in mice exposed to alcohol compared to the control mice (P<0.001) (Figure 3E), while trigonelline treatment at both doses could diminish (P<0.001) alcohol-induced up-regulation of NF-κB.

Additionally, NF-κB activation was detected by immunofluorescence staining of p-NF-κB p65 in the brain tissues (Figures 3F and G). The results revealed that in mice exposed to alcohol, nuclear p-NF-κB staining (green) was remarkably higher than that in the control mice (P<0.001). This finding indicates that alcohol could increase the activation of NF-κB in the brain cells. However, the levels of p-NF-κB were significantly diminished in the trigonelline-treated groups (P<0.001).

To further confirm whether anti-inflammatory

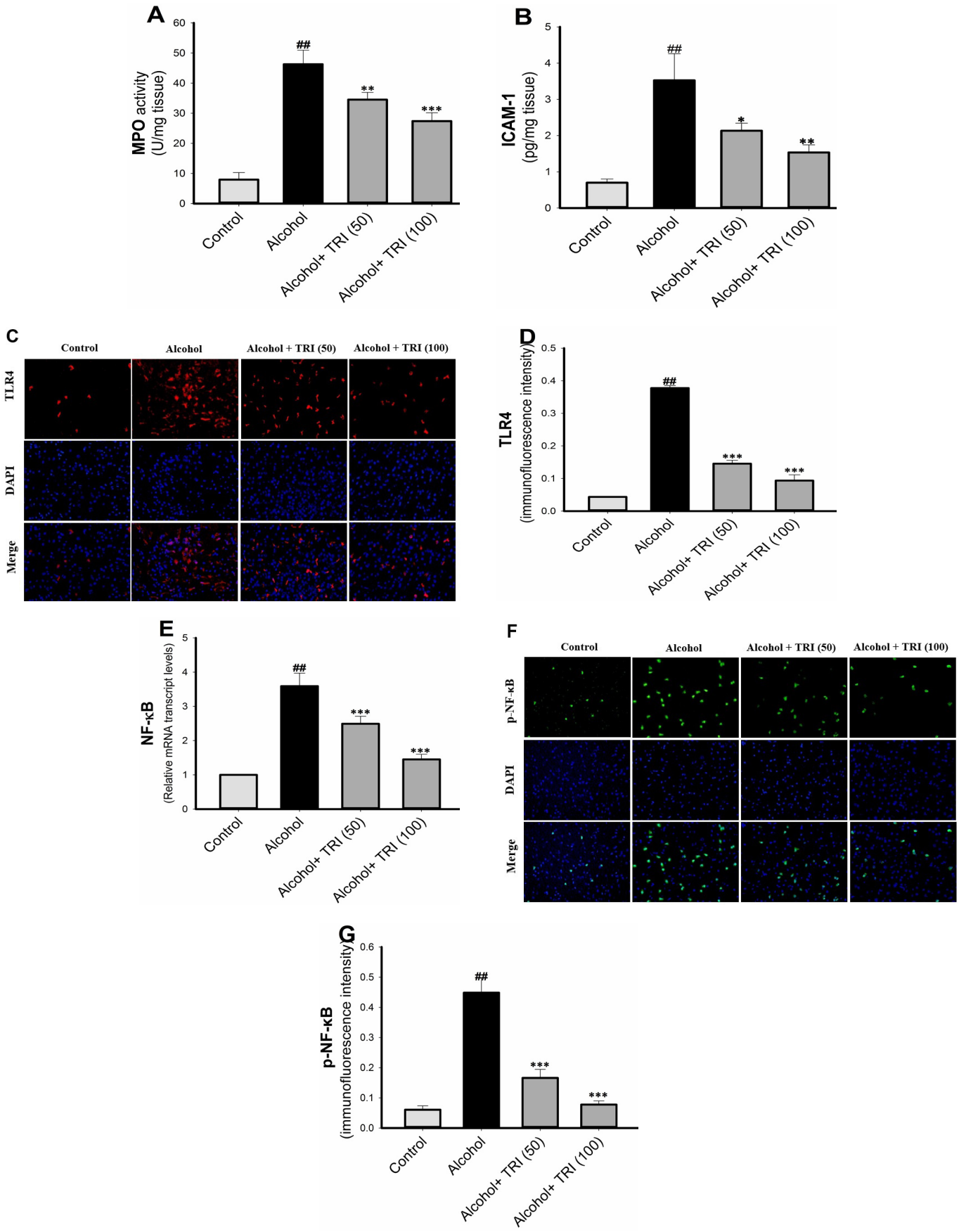

Figure 3. Effect of trigonelline on the levels of MPO (A), ICAM-1 (B), immunofluorescence expression of TLR4 (C, D), mRNA levels of NF-κB (E), and immunofluorescence expression of p-NF-κB (F, G) in the brain of mice

Treatment with trigonelline (50 and 100 mg/kg) significantly decreased the levels of MPO, ICAM-1, and the activity of TLR4 and NF-κB in the brain of mice exposed to alcohol. Data are presented as mean ± SD (n = 4). ##P<0.001 (compared with control group), *P<0.05, **P<0.01, ***P<0.001 (compared with alcohol group).

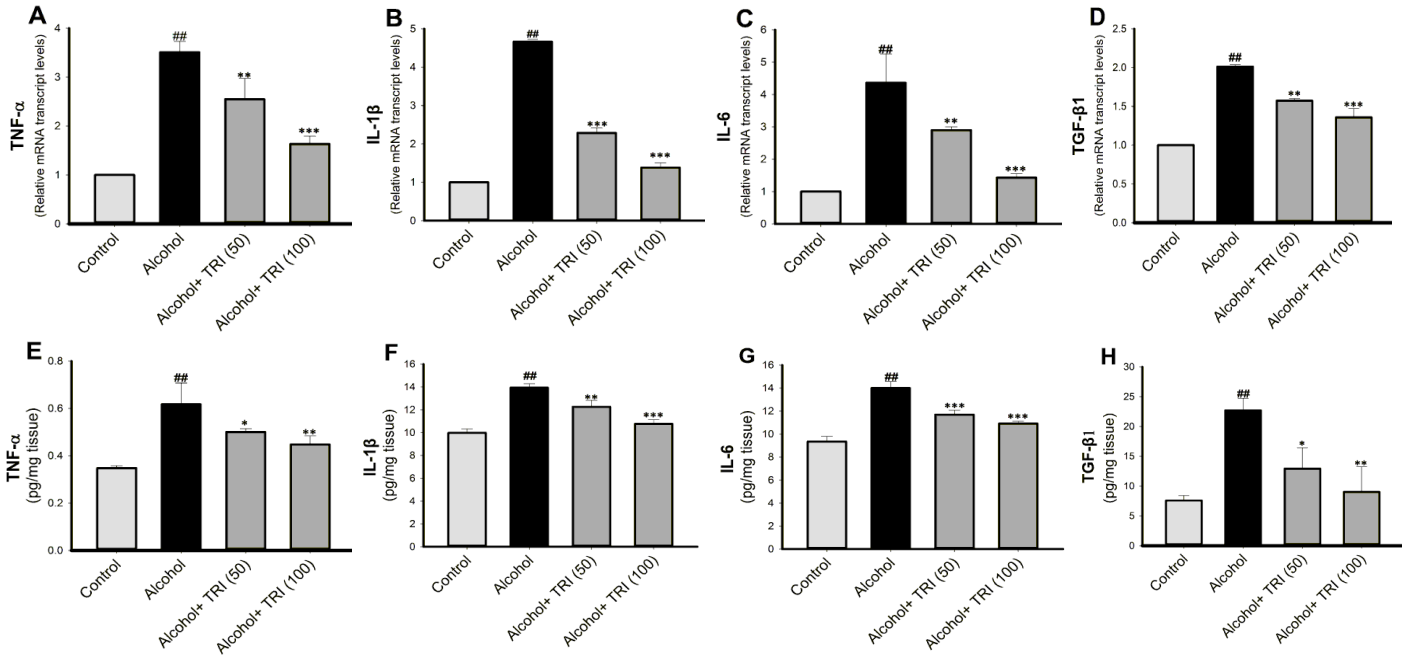

Figure 4. Effect of trigonelline on the mRNA expression (A-D) and the brain levels (E-H) of TNF-α, IL-1β, IL-6, and TGF-β1 treatment with TRI (50 and 100 mg/kg) decreased the expression and brain tissue levels of these pro-inflammatory cytokines in the mice exposed to alcohol. Data are presented as mean± SD (n= 4). ##P<0.001 (compared with control group), *P<0.05, **P<0.01, ***P<0.001 (compared with alcohol group)

mechanisms were involved in the protective effect of trigonelline against toxic effects of alcohol in the brain, we detected the levels of pro-inflammatory cytokines. As shown in Figure 4A–D, administration of alcohol in mice resulted in a significant elevation (P<0.001) in the mRNA expression of TNF-α, IL-1β, IL-6, and TGF-β1 in the brain as compared with those in the control mice. However, treatment with trigonelline significantly diminished the expression of alcohol-induced proinflammatory cytokines TNF-α (P=0.002 at 50 mg/kg, P<0.001 at 100 mg/kg), IL-1β (P<0.001 at 50 and 100 mg/kg), IL-6 (P=0.005 at 50 mg/kg, P<0.001 at 100 mg/kg) and TGF-β1 (P<0.001 at 50 and 100 mg/kg), compared with those in the alcohol group.

Moreover, assessment of the brain content of these cytokines revealed that exposure to alcohol resulted in a significant elevation (P<0.001) in the brain levels of TNF-α, IL-1β, IL-6, and TGF-β1, compared with those in the normal control mice (Figure 4E–H). Treatment of mice with trigonelline at both doses significantly reduced the brain levels of TNF-α (P<0.05 at 50 mg/kg, P=0.002 at 100 mg/kg), IL-1β (P=0.006 at 50 mg/kg, P<0.001 at 100 mg/kg), IL-6 (P<0.001 at 50 and 100 mg/kg), and TGF-β1 (P<0.05 at 50 mg/kg, P=0.005 at 100 mg/kg) as compared with those in the alcohol group.

Trigonelline inhibits apoptosis induced by high-dose alcohol

Since apoptosis has been offered as a potential mechanism of ethanol-induced brain injury, the anti-apoptotic properties of trigonelline were examined in this study. Administration of high-dose alcohol (6 g/kg) resulted in a significant increase in the levels of caspases-3 and cytochrome c in the brain of mice as compared with the control group (Figures 5A and B). This result was aligned with TUNEL staining data (Figures 5C and D) which revealed that the percentage of apoptotic cells was significantly augmented in the brain of alcohol-exposed mice as compared to the control mice (P<0.001). Trigonelline treatment decreased the levels of caspases-3 (P=0.015 at 50 mg/kg, P<0.001 at 100 mg/kg), cytochrome c (P=0.016 at 50 mg/kg, P<0.001 at 100 mg/kg) and TUNEL positive cells (P<0.001 at 50 and 100 mg/kg) in the brain of alcohol-treated mice. These results suggest that trigonelline suppresses alcohol-induced apoptosis in brain cells through inhibition of caspase-3 and cytochrome c.

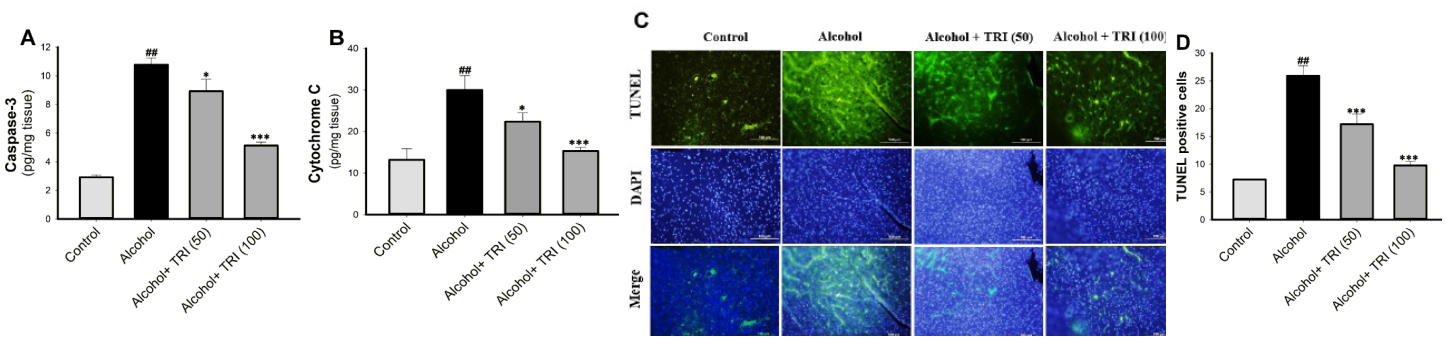

Figure 5. Effect of trigonelline on the brain levels of caspases-3 (A) and cytochrome c (B) in mice. Treatment with TRI (50 and 100 mg/kg) decreased the brain levels of these pro-apoptotic markers in the mice exposed to alcohol. Representative TUNEL fluorescence images (C), and the percentage of TUNEL-positive cells in the brain (D).
Trigonelline significantly decreased the number of alcohol-induced apoptotic cells. Data are presented as mean±SD (n=3-4). ##P<0.001 (compared with control group), *P<0.05, ***P<0.001 (compared with alcohol group)

Discussion

Brain damage is the serious complication of alcohol consumption at high doses. The present study was demonstrated that trigonelline may protect the brain cells against alcohol intoxication through various mechanisms.

Oxidative stress is thought to play an important role in alcohol-induced brain damage leading to the behavioral and cognitive impairments. Numerous evidences suggest that antioxidant imbalance is implicated in the pathophysiology of brain injury following alcohol intoxication. Alcohol metabolism in the brain generates high levels of reactive oxygen and nitrogen species, which can react with cellular biomolecules, including polyunsaturated fatty acids and lead to lipid peroxidation and subsequent cell membrane damage (2, 6).

Clinical and experimental studies have shown that ethanol exposure especially at higher doses depresses the activity of cytosolic antioxidant protective enzymes, including SOD, catalase, and GSH as a potent thiol containing antioxidant. Reduction in the levels of cellular antioxidants in the CNS results in the accumulation of oxygen species such as superoxide anion and H_2O_2, which are highly toxic to cellular proteins, lipids and nucleic acids (29, 30). It has been well known that compounds with antioxidant properties are able to inhibit neurotoxicity and prevent binge ethanol-induced brain injury (31).

Consistent with previous studies, the results of our study also demonstrated that mice subjected to alcohol showed a remarkable increase in lipid peroxidation and oxidized protein levels in the brain. Moreover, low levels of GSH, HO-1, and SOD in the brain of mice exposed to high-dose alcohol may indicate that alcohol-induced overproduction of oxidative mediators led to the depletion of these antioxidant markers. This event can decrease the defense mechanism of cells against oxidative damage and consequently impair the normal function of the brain. Administration of trigonelline (50 and 100 mg/kg) reduced lipid peroxidation procedure and maintained the levels of antioxidants GSH, HO-1, and SOD in the brain of mice exposed to high-dose alcohol. Indeed trigonelline could improve the ability of cells to protect themselves against alcohol-induced ROS. This finding could demonstrate the potent antioxidant property of trigonelline.

In consistence with our study, it has been reported that trigonelline as a bioactive compound of fenugreek protects heart tissue against oxidative damage in alcohol intoxicated rats. This compound is able to scavenge toxic mediators such as hydroxyl radical and H_2O_2 and reduce MDA levels (17). Trigonelline has been shown to protect different brain regions from LPS and oxygen-glucose deprivation-induced oxidative damage through increasing the activity of SOD and GSH (20, 32).

It has been shown that systemic administrations of ethanol and its primary metabolite acetaldehyde dose-dependently increased NO levels in the brain of rats. NO is a signaling molecule and free radical which can be excessively produced in the CNS by iNOS enzyme in oxidative and inflammatory pathways (33). High levels of NO induced by ethanol can contribute in neurotoxicity through the formation of oxidative radicals such as peroxynitrite and activation of inflammatory reactions. It has been shown that inhibition of iNOS/NO signaling pathway may protect brain cells from ethanol-induced brain injury (2, 34). In line with the results of these studies, we also found that administration of alcohol increased the expression of iNOS and consequently NO levels in the brain of mice. Inhibition of iNOS/NO system by trigonelline treatment significantly reduced alcohol-induced brain damage. This result proposes that trigonelline can protect brain tissue against toxic effects of NO by inhibiting the overproduction of NO in the cells.

Human and animal studies have demonstrated that excessive alcohol consumption promotes pro-inflammatory mediators in the CNS, leading to brain inflammation and ultimately alcohol-related cognitive and behavioral dysfunctions (11, 35). MPO is a peroxidase enzyme found in neutrophils and released during inflammation. Chronic ethanol intake may promote immune cell infiltration in the CNS and increase MPO activity in the brain (36). ICAM-1 is an inflammatory adhesion molecule that plays a main role in brain inflammation and its concentration can be increased by inflammatory cytokines such as TNF-α. ICAM-1 mediates the activation and transfer of immune cells into the brain during CNS inflammatory conditions (37). It has been reported that the effect of alcohol on ICAM-1 is dose dependent. Low doses of alcohol may decrease the expression of ICAM-1, whereas at higher doses alcohol increases ICAM-1 levels and exerts an inflammatory effect (38).

The findings of the present study also revealed that the brain levels of ICAM-1 and MPO were augmented by high-dose alcohol. Trigonelline treatment reduced the levels of ICAM-1 and MPO as markers of inflammatory events in the brain exposed to alcohol. Consistent with this finding, it has been reported that trigonelline (at 100 mg/kg) displays neuroprotection in the rat model of ischemic stroke through the reduction of glutathione mediated MPO expression in the cortical region of the brain (21).

The TLR4/NF-κB signaling pathway is a key regulator of the immune system that can play an imperative role in the development of inflammation. Inappropriate activation of this pathway in the CNS may trigger the excess release of several inflammatory mediators which contribute to neurodegeneration. It has been shown that alcohol-induced neuroinflammation and brain injury are linked to the activation of TLR4 receptors in the brain, mainly in glial cells. Stimulation of TLR4 triggers the signaling pathways that lead to the induction of transcription factor NF-κB and subsequently the gene expression of proinflammatory cytokines, such as IL-6, TNF-α, and IL-1β. NF-κB can regulate cellular activities in response to various detrimental stimuli such as free radicals, LPS, and alcohol (9, 36, 39). NF-κB induces the expression of iNOS and cyclooxygenase 2 (COX-2) and promotes the release of NO and prostaglandins, which are major inflammatory mediators. Inhibition of NF-κB activity has been shown to protect against ethanol-induced neuroinflammation and brain injury (2, 40).

According to the findings mentioned above, we thought that targeting TLR4/NF-κB signaling pathway can be a therapeutic approach for the treatment of alcohol-induced brain injury. The results of the present work also demonstrated that administration of alcohol increased the expression of TLR4 and the activity of NF-κB in the brain of mice. Treatment with trigonelline significantly prevented ethanol-induced activation of TLR4/NF-κB pathway in the brain of mice. Indeed, suppressing TLR4/NF-κB may be

the main mechanism of trigonelline against inflammation because activation of this pathway prompts the expression of other inflammatory mediators such as cytokines. In agreement with this finding, it has been reported that trigonelline exerts neuroprotective and memory improvement properties by modulating NF-κB and TLR4 signaling in LPS-induced neuroinflammation in the brain of rats (20).

As a cytoprotective enzyme, HO-1 is found in neuronal cells and microglia and exhibits important antioxidant and anti-inflammatory functions (41). It has been shown that microglial HO-1 is necessary to reduce TLR4-mediated neuroinflammation, neuronal apoptosis and brain injury in a mouse model of subarachnoid hemorrhage. The protective effect of HO-1 is mediated by enzymatic degradation of heme which can bind to TLR4 and triggers the expression of NF-κB, proinflammatory cytokines and ICAM-1 (42).

In the present study, we observed that trigonelline up-regulated HO-1 and alleviated TLR4 and its downstream inflammatory mediators in the brain exposed to alcohol. Therefore, we hypothesized that induction of HO-1 by trigonelline might be a therapeutic approach in alcohol-induced brain injury.

To further investigate the downstream inflammatory mediators regulated by TLR4/NF-κB, we analyzed the levels of proinflammatory cytokines in the brain tissue. Our data demonstrated that administration of high-dose alcohol resulted in a remarkable increase in the gene expression and production of IL-1β, IL-6, TNF-α, and TGF-β1 in the brain. Treatment with trigonelline inhibited the elevation of these proinflammatory cytokines in the brain of mice intoxicated by alcohol. Down-regulation of proinflammatory cytokines appears to be another possible mechanism of trigonelline against alcohol-induced inflammation in the CNS.

These findings are in agreement with previous studies showing the key role of cytokines in neurotoxicity related to alcohol. It has been reported that binge alcohol exposure (5 g/kg) can increase the levels of IL-6 in the cerebellum and IL-1β in the cerebral cortex of mice (6). Binge dose of alcohol (5 g/kg) also enhances the level of TNF-α as a critical proinflammatory cytokine which can activate inflammatory cascades in the CNS by promoting the production of other cytokines and chemokines (2, 43). Excessive ethanol use may increase the expression of IFN-γ by the activation of astrocytes and lead to IFN-γ-induced neurotoxicity in human brain (44). Alcohol induces the expression of TGF-β1 which is an effective immunoregulatory cytokine and has a significant role in neuroinflammation and apoptosis. Excess production of TGF-β1 impairs antioxidant pathways in the brain and may lead to neuronal disruption and cognitive impairment (45).

Animal and clinical studies have shown that exposure to alcohol activates apoptotic signaling through induction of oxidative and inflammatory cascades. Ethanol-induced oxidative damage to the permeability of mitochondrial membrane can initiate the release of intracellular apoptotic molecules such as caspases and cytochrome c. Ethanol-induced neurodegeneration has been proven to be associated with significant up-regulation of cytochrome c and caspase-3 as proapoptotic markers (7, 46). TLR4 signaling is an important mechanism of alcohol-induced apoptosis in the CNS. TLR4 is expressed in various brain cells, including neurons, microglia and astrocytes, and regulates inflammation-mediated neuronal death. Alcohol activates TLR4 signaling in glial cells and astrocytes and increases the release of oxidative molecules and cytokines such as IL-1β and TNF-α, which are the major mediators of apoptosis (9). It has also reported that TUNEL-positive cells as indicator of apoptosis were increased in the brains of human alcoholics (47).

In the present study, we also observed an increase in the levels of caspase-3 and cytochrome c, indicating enhanced apoptotic process in the brain of mice exposed to alcohol. Our data revealed that administration of high-dose alcohol increased TUNEL-positive cells as a marker of apoptotic DNA-damaged cells in the brain of mice. While, trigonelline treatment reduced these apoptotic markers in the brain tissue. As a result, trigonelline exerts a significant anti-apoptotic effect against alcohol-induced toxicity and cell death in the CNS. In line with our finding, trigonelline has been shown to protect hippocampal neurons from cell apoptosis by suppressing caspase-3 activity in an experimental model of cerebral injury (22).

Conclusion

Consumption of alcohol at high doses induces neurotoxicity and brain injury. The results of the present study suggests that trigonelline shows therapeutic effect against ethanol-associated neurotoxicity. The underlying mechanisms might be related to the restoration of antioxidant defense, inhibition of TLR4/NF-κB-mediated inflammatory responses, and reduction of apoptosis in the brain. The limitations of this study are the absence of further behavior tests and histological (H&E) evidences. Nevertheless, further clinical experiments are needed to validate these data and evaluate the effectiveness of trigonelline against alcohol-induced CNS toxicity in humans.

Acknowledgment

This study was financially supported by Ardabil University of Medical Sciences, Ardabil, Iran.

Conflicts of Interest

No conflict of interest.

Authors' Contributions

K A designed the experiments, supervised, and directed and managed the study; A N and M I performed experiments, analysis, and interpretation of results.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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